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METHOD OF CONTROLLING PLANT PESTS

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(57) Claim

1. A method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein:

(a) said inhibitor is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases with the proviso that the serine proteinase inhibitor is not a member of the Bowman-Birk inhibitor family;

(b) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(c) said plant is: (i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or (ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

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29. A method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein said inhibitor:

(a) is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(b) is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases.

63. A plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, the proteinase inhibitor being selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

90. A transgenic plant containing a gene capable of expressing a proteinase inhibitor, or a gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, selected from the group consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, Drug Crops and Spices and Flavorings.

104. A DNA sequence coding for chicken egg white cystatin wherein the codons are those preferred in corn.

106. A vector comprising a DNA sequence coding for a proteinase inhibitor wherein the vector is useful for transformation of plant cells or of *Agrobacterium*.

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Complete Specification for the invention entitled:

Method of Controlling Plant Pests

The following statement is a full description of this invention, including the best method of performing it known to me/us

5845/3

Abstract of the Disclosure

The present invention relates to a method of controlling a plant pest based on proteinase inhibitors which are synthesized in the plant as a result of genetic manipulation. It also relates to transgenic monocot and dicot plants containing genes which encode such proteinase inhibitors or protein precursors thereof and plants which express these genes and to a method of controlling plant pests by exposing them to such a transgenic plant. Another object are DNA-sequences and vectors comprising these sequences coding for proteinase inhibitors.

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Method of Controlling Plant Pests

The present invention relates to a method of controlling pests on plants. More particularly, it relates to a method of controlling pests on plants by exposing the pests to proteinase inhibitors which are biologically synthesized by the plants. The invention also relates to plants capable of exhibiting such control.

A. Proteinases and their Inhibitors

A proteinase is conventionally regarded as an enzyme which hydrolyzes peptide bonds, thereby destroying molecules, such as proteins, which contain such bonds. If the proteinase severs a peptide bond of a terminal amino acid, it is designated an "exoproteinase". If the enzyme hydrolyzes a non-terminal peptide bond, it is designated an "endo-proteinase". However, for purposes of the present invention, the term is also considered applicable to materials which sever peptide bonds by mechanisms other than hydrolysis.

Both classes of proteinase exhibit different modes of action. Exoproteinases may cleave at either the N-terminus or the C-terminus of the peptide. Aminopeptidases are ones acting at the N-terminus while carboxypeptidases are ones attacking the C-terminus.

Endoproteinases are more specific in their activities. By convention these enzymes are categorized in four principal classes reflecting the nature of the hydrolyzed bonds and/or the cavity surrounding the hydrolysis site and/or the requirement of a characteristic moiety in the proteinase.

Serine endoproteinases are characterized by the participation of a serine hydroxyl group in the hydrolysis reaction of the protein being deactivated. As a group these proteinases are probably the best characterized. However, they appear to be far more prevalent in microbial tissues and animal tissues than in plant tissues.

Thiol proteinases, or sulfhydryl proteinases, appear to be the most prevalent kind of plant tissue proteinases. They are characterized by the apparent involvement of sulfur in some form in the hydrolysis reaction. A free sulfhydryl group on cysteine has been identified in the active site of a number of these proteinases.

The acidic proteinases, also known as carboxylic proteinases, are widespread throughout the plant and animal kingdoms. Active sites appear to contain two aspartic acid side chains which share a common proton and a hydroxyl group from a tyrosine residue.

The metalloproteinases, for purposes of the present discussion, are generically defined as those proteinases which do not fall into one of the above three groups and which require at least one metal ion for their hydrolytic activities. Calcium, zinc and iron are among the more common metals found in such proteinases.

Proteinase inhibitors are materials which destroy or limit the activity of proteinases. A variety of classification schemes has been developed to correlate the activities of particular inhibitors with their target proteinases and/or other inhibitors. The predominant one correlates the activity of the inhibitor with that of the proteinase. Thus, the four principal classes of proteinase inhibitors are serine proteinase inhibitors, thiol proteinase inhibitors, acidic proteinase inhibitors and metalloproteinase inhibitors.

Serine proteinase inhibitors are found naturally in several kinds of tissues in many plants. They appear to be active against a wide variety of serine proteinases from both insects and microorganisms. Evidence

suggests that the target proteinase is deactivated by the formation of a stable complex between proteinase and inhibitor, a complex which is incapable of hydrolyzing peptides.

Inhibitors of thiol proteinases, acidic proteinases and metalloproteinases are less well understood from a mechanistic point of view. They are found throughout the plant kingdom, although they appear to be less ubiquitous than the serine proteinase inhibitors. It is thought that the inhibitors of thiol proteinases act primarily through a mechanism in which the characteristic sulfur moiety is blocked.

Proteinase inhibitors may also be classified according to structural considerations. A variety of "low molecular weight" proteinase inhibitors are known, largely of non-natural synthetic origin, and are useful as laboratory reagents. A number of naturally occuring low molecular weight inhibitors have been isolated from bacterial and fungal sources and characterized; this group includes such inhibitors as the leupeptins, antipains and pepstatins. A large number of naturally occurring proteinase inhibitors are actual proteins which are produced directly from gene expression in the cell, or synthesized in a series of chemical reactions which are controlled by enzymes that are produced directly from gene expression in the cell.

B. Proteinase Inhibitors in Plants

Plant proteinase inhibitors are commonly found in plant tissues, apparently as a part of a biological control mechanism to protect the plant against attack by a variety of pests, particularly insects or microorganisms. In response to such attack, it is thought that the plant releases one or more proteinase inhibitors which, when ingested by the attacker, deactivate(s) proteinases within the body of the attacker. Such deactivation probably interferes with the metabolic processes of the attacker, particularly its digestive processes, thus slowing or terminating its metabolism.

It has been shown that the addition of proteinase inhibitors to the diets of some insects inhibits growth of certain insects. For example, Gatehouse and Boulter (1983) have shown that cowpea *(Vigna unguiculata)* trypsin inhibitor decreases the development of cowpea weevil *(Callosobruchus maculatus)* larvae; however, soybean *(Glycine max)* and lima bean *(Phaseolus lunatus)* inhibitors are much less effective. Murdock et al. (1987) have demonstrated that some Coleopteran insects experience growth retardation when exposed to inhibitors of thiolproteinases.

These results indicate that naturally occurring proteinase inhibitors provide a potent means to control insect or microbial pests on plants. However, large scale use of naturally occurring inhibitors suffers from a number of disadvantages. Such compounds are typically proteinaceous and, therefore, are expensive to isolate (or otherwise produce) in large quantities, to purify and to formulate. Because many are readily degraded in the environment, activity is short-lived, requiring multiple applications. In addition to degradation, many such proteinase inhibitors are water soluble and thus wash away after the first rain storm or watering.

However, of perhaps even greater concern is the non-specificity of many proteinase inhibitors. That is, many inhibitors exhibit activity against a variety of insects and/or microorganisms. Accordingly, use of a particular inhibitor against a particular pest may very well have an adverse impact upon beneficial insects and/or microorganisms.

C. In Vivo Synthesis of Proteinase Inhibitors in Plants

As the field of molecular biology has developed, control of the genetic processes leading to biological production of proteinaceous materials in a wide variety of biological organisms is now routine in many instances. Of particular interest in the development of genetic engineering, or recombination DNA technology, has been the transplanting of genes from one organism into another that is quite different in its properties, thus endowing the receiving organism with phenotypes not characteristic of that organism.

The transformation of plants has developed more slowly than transformation of other eukaryotic organisms. However, in recent years techniques to transform plant tissue and regenerate whole, fertile plants have become more numerous and reliable. Therefore, the possibility of transforming plants to express particular proteins is now an objective that can be realized effectively and in a reasonably efficient manner.

In recognition of the advances of recombinant DNA technology, and of the disadvantages associated with the use of proteinase inhibitors in a pure, or substantially pure form, it now becomes possible to develop transgenic plants which are capable of biologically synthesizing proteinase inhibitors which provide the plants with a new, or an additional, mechanism to protect themselves from attack by insects and/or microorganisms making it unnecessary to apply exogenous chemicals.

The disadvantages outlined previously for the use of substantially pure proteinaceous proteinase inhibitor are entirely or substantially overcome by such an approach. The inhibitor is produced in pure form in sufficient amount, and there is no need to apply or formulate it. Environmental degradation and loss become minimal if not irrelevant. And the biological synthesis of a particular proteinase inhibitor permits some control over the specificity of the inhibitor used in the plant of interest.

The possibility of endowing plants at the genetic level with the ability to express a foreign proteinase inhibitor, that is, a proteinase inhibitor which it does not normally express, has recently been demonstrated (Hilder et al., 1987). In this work tobacco plants were transformed with the gene which encodes cowpea trypsin inhibitor, thus imparting to tobacco resistance to *Heliothis virescens*.

Clearly this demonstration is quite informative. However, tobacco is a model system which is easily transformed, and is therefore widely used for introductory experiments in plant transformation. The present invention is directed to the transformation of a variety of plants, both for purposes of model studies and commercial utility, with genes for a variety of proteinase inhibitors.

The variety of plants and inhibitors embraced by the present invention is extensive. The transformation of tobacco with genes encoding non-trypsin proteinase inhibitors is of specific interest for its value as a model. However, the transformation of a variety of monocot and dicot plants with widespread utility and substantial commercial benefit represents a significant advance, the benefits of which span the entire realm of agriculture. That is, the development of plants with increased resistance to pests has obvious advantages to the growers and users of plants useful as food, forage, ornamental, fiber, energy and pharmaceutical resources.

SUMMARY OF THE INVENTION

A principal object of this invention is a transgenic plant which exhibits as a phenotype the ability of in vivo synthesis of a foreign proteinase inhibitor, preferably in an amount effective to control a particular pest, thus imparting to the plant new or improved resistance to that pest.

A corollary object is the development of new transgenic monocot plants showing such a phenotype.

Another corollary object is the development of new transgenic dicot plants showing such a phenotype.

Another principal object of this invention is a method of controlling plant pests by exposing them to such a transgenic plant.

According to a first embodiment of this invention, there is provided a method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein:

(a) said inhibitor is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases with the proviso that the serine proteinase inhibitor is not a member of the Bowman-Birk inhibitor family;

(b) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(c) said plant is: (i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar

Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or (ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

According to a second embodiment of this invention, there is provided a method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein said inhibitor;

(a) is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(b) is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases.

According to a third embodiment of this invention, there is provided a plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, the proteinase inhibitor being selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

According to a fourth embodiment of this invention, there is provided a transgenic plant containing a gene capable of expressing a proteinase inhibitor, or a gene or gene group capable of expressing one

or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, selected from the group consisting of inhibitors or thiol-proteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Proteins Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, Drug Crops and Spices and Flavorings.

According to a fifth embodiment of this invention, there is provided a DNA sequence coding for chicken egg white cystatin wherein the codons are those preferred in corn.

According to a sixth embodiment of this invention, there is provided a vector comprising a DNA sequence coding for a proteinase inhibitor wherein the vector is useful for transformation of plant cells or of *Agrobacterium*.

Brief Description of the Figures

Fig. 1 describes the construction of pRK252/Tn903/BglII.

Fig. 2 shows the construction of pCIB5.

Figs. 3 and 4 illustrate the construction of pCIB4.

Fig. 5 discloses the construction of pCIB2.

Fig. 6 contains the construction of pCIB10, a broad host range plasmid containing T-DNA borders and gene for plant selection.

Fig. 7 describes the construction of pCIB710.

Fig. 8 reports the construction of pCIB10/710.

Fig. 9 contains the sequence of a cystatin gene which is incorporated into a transgenic plant according to this invention.

Fig. 10 discloses the synthetic fragments which are prepared and then assembled to produce the cystatin gene of Figure 9.

Fig. 11 contains the synthetic fragments which are prepared and then assembled for the soybean Kunitz trypsin inhibitor gene.

Brief Description of Tables A and B

Tables A contains a listing of plants according to their uses. It has been extracted from Christie (1987).

Table B illustrates representative transgenic plants containing genes for proteinase inhibitors, or precursors for proteinase inhibitors, prepared in accordance with this invention and representative insects and other pests which may be controlled by these plants. It is intended in no way to limit the scope of the invention as described and claimed herein.

DETAILED DESCRIPTION OF THE INVENTION

A. Generic Aspects of the Invention

In a generic aspect the present invention is directed to a method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein:

(a) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

In a second generic aspect the present invention is also directed to a method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein said inhibitor:

(a) is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(b) is selected from the group of non-trypsin proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases.

More specifically, the present invention includes a method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein said inhibitor:

(a) is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(b) is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases.

In a further aspect the present invention embraces a transgenic plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or one or more precursors for a proteinase inhibitor, the proteinase inhibitor being selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

In another aspect the present invention embraces a transgenic plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:

(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or one or more precursors for a proteinase inhibitor, the proteinase inhibitor being selected from the group consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases; and

(b) said plant is:

(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or

(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, Drug Crops and Spices and Flavorings.

Furthermore the present invention is directed to transgenic plants expressing a proteinase inhibitor useful in the mentioned method of controlling a pest which is attacking a target plant.

B. Definitions

In order to provide a clear and concise understanding of the specification and the claims, including the scope given to the language in each, the following definitions are provided:

Biological Synthesis of a Proteinase Inhibitor: Synthesis of a proteinase inhibitor in a host cell. This term is intended to embrace: (1) processes in which an active proteinaceous proteinase inhibitor is produced as a result of expression of a gene encoding that proteinase inhibitor; (2) processes in which a translation product is modified by enzymatic action post-translationally; and (3) processes in which one or more proteins are produced as a result of expression of one or more genes, the proteins participating in a reaction scheme which converts one or more proteinaceous or non-proteinaceous precursors to an active proteinase inhibitor. This last process specifically embraces, but is not

limited to, the situations in which a material exogenous or endogenous to the plant is converted by such a reaction scheme to an active proteinase inhibitor.

Biological Synthesis as a Result of Gene Expression: Synthesis in a host cell of a product that results directly from gene expression (that is, as a product of translation) or synthesis of a product that results indirectly from gene expression (that is, as a product of chemical reactions carried out by translation products).

Control of a Pest: The killing of a pest or the inhibition of activity deleterious to a host by that pest.

Foreign Gene or Gene Group: A gene or group of genes which originates from a host different from that which ultimately exhibits the desired phenotype; or which originates from the host but which has been modified in some fashion. Modification in this latter situation specifically includes, but is not limited to, any change in the DNA sequence of the gene or any addition of one or more coding sections which encode an additional phenotype (such as an antibiotic marker trait).

Gene: A DNA sequence containing any segments necessary to effect translation of a protein. The necessary segments include at least a promoter sequence, a coding sequence and a terminator signal. However, those segments do not necessarily originate from the same source; that is, the term "gene" as used herein embraces DNA segments which originate from the same or different sources.

Non-trypsin proteinase: A proteinase whose mode of action differs substantially from that of a trypsin according to conventional classification schemes or the understanding of one skilled in the art.

The term specifically embraces, but is not limited to, proteinases having two or more modes of action, the most significant of which differs substantially from that of a trypsin, although a less or the least significant of which may be like that of a trypsin. For purposes of this

invention, an inhibitor of this kind of non-trypsin proteinase is one which interferes with at least the primary mode of action, irrespective of any interference with the trypsin mode of action.

Pesticidally effective amount: An amount sufficient to achieve control of a pest.

Plant: A plant in the conventional sense of the term as well as plant tissue in a plant or grown, for example, in a tissue culture medium.

Precursor of a proteinase inhibitor: A material, proteinaceous or non-proteinaceous, which is converted enzymatically to an active proteinase inhibitor in the host cell, or which participates (either as a reactant or as an enzyme) in the synthesis of a proteinase inhibitor. The term specifically embraces, but is not limited to, a single compound or a group of compounds which is or are endogenous or exogenous to a host cell. The term also specifically embraces one or more enzymes participating in a pathway to synthesize a proteinase inhibitor from cellular substrates; in this situation both the substrate and the enzyme are considered as precursors of a proteinase inhibitor.

Proteinase: A material, either a unique compound or a group of compounds operating in concert, which deactivates a protein. Although the term "proteinase" is used interchangeably with "protease" herein, "proteinase" is the term of predominant usage.

Proteinase inhibitor: A material, either a unique compound or a group of compounds operating in concert, which deactivates a proteinase.

Transgenic plant: A plant containing at least one DNA sequence, which may or may not be a gene, which is different from that occurring in the analogous wild-type plant. As used herein the term embraces any plant in which any DNA has been modified relative to the DNA of the wild-type plant. However, there is no requirement that the modified DNA endow a new phenotype relative to the wild-type plant. The term also embraces plants

containing an increased number of gene copies relative to the wildtype and plants containing any modified DNA sequences not necessarily restricted to those which encode proteins.

Functional Similarity of Inhibitors: Inhibitors have functional similarity if they can be exchanged by each other without a significant loss of action, i.e. if they show an action of the same kind and if their effectiveness is in the same order of magnitude.

C. Transgenic Plants and Method of Pest Control

(1) Transgenic Plants Containing, and Use of, Proteinase Inhibitors of All Four Classes

The present invention provides a means of controlling pests on a number of transgenic target monocot and dicot plants using proteinase inhibitors which are representative of all four conventional classes of those inhibitors and which are biologically synthesized in those plants. Pests that can be controlled in this manner are predominantly insects, acari, fungi or bacteria.

Plants of particular interest to this aspect of the present invention include: (a) monocots selected from the group of plant types consisting of Ornamentals and those listed in Table A as Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods, and Spices and Flavorings; and (b) dicots selected from the group of plant types consisting of Ornamentals and those listed in Table A as Cereals, Proteins Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings. That is, dicots of the Drug Crops are not included in this aspect of the invention.

Preferred monocot plant types include those of the Cereals, Vegetables and Tubers, Sugar Crops and Forage and Turf Grasses. Particularly preferred are plants of the genera *Avena* (oats), *Hordeum* (barleys), *Oryza* (rice), *Sorghum* (sorghum), *Triticum* (wheats), *Dactylis* (cocksfoot,

orchard grass) and *Saccharum* (sugar cane), and *Zea mays* (maize). Most preferred are plants of the genus *Dactylis* and *Zea mays*, in particular *Zea mays*.

Preferred dicot plant types include those of the Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes and Fiber Plants and Woods. Particularly preferred are plants of the genera *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales) and *Gossypium* (cottons). Most preferred are plants of the genera *Lycopersicon*, *Solanum* and *Gossypium*. Another group of preferred plants consists of potato, rape, tomato, soybean, pea and cotton.

Inhibition may be observed in transgenic plants containing a foreign gene or gene group encoding an inhibitor of a serine proteinase. Preferably that inhibitor is an inhibitor of at least one serine proteinase selected from the group consisting of thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, $C\bar{1}$-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent protease, thermitase, mast cell proteinase I and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio* α-proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, or of a proteinase which has substantial structural or functional similarity to any of these.

In addition, the inhibitor may be an inhibitor of trypsin or chymotrypsin. When the proteinase is chymotrypsin, the inhibitor is preferably a potato I, potato II, tomato I or tomato II inhibitor.

Inhibition is also observed as a result of in vivo synthesis of other inhibitors of serine proteinases when one or more of such inhibitors is a member of the Bowman-Birk inhibitor family, the soybean Kunitz inhibitor family, the bovine pancreatic trypsin (Kunitz) inhibitor family, the Kazal trypsin inhibitor family, the *Streptomyces* subtilisin inhibitor family, the potato inhibitor I family, the potato inhibitor II family, the α_1-proteinase inhibitor family, the hirudin family, the bdellin

family, the eglin family, the inter-α_1 trypsin inhibitor family, the serpin superfamily, the $C\bar{1}$-inhibitor family, the *Ascaris* inhibitor family, the leupeptins, the antipains, elastinal and chymostatin, or an inhibitor which has substantial structural of functional similarity to any of these.

Particularly preferred serine protease inhibitors are soybean Kunitz trypsin inhibitor, α_1-antitrypsin (an example of the α_1-proteinase inhibitor family), eglin C and eglin C mutants, especially eglin C (Arg 45).

A preferred group of inhibitors biologically synthesized in a transgenic plant and useful in controlling pests, particularly insect pests, contains an inhibitor of thiolproteinase. Preferred examples of such inhibitors include inhibitors of papain, bromelain, ficin, calpain, cathepsin B, cathepsin C, cathepsin L, cathepsin H, cathepsin S, chymopapain, clostripain, asclepain, prolyl endopeptidase, pyroglutamyl peptidase, dipeptidyl proteinase I, yeast proteinase B, *Streptococcus* proteinase, *Staphylococcus* thiol proteinase or actinidin, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

Particularly preferred is an inhibitor which is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64 or a derivative thereof. E64 is a simple name used to designate the compound [N-(L-3-transcarboxyoxiran-2-carbonyl)-L-leucyl]-amido(4-guanido)-butane. Derivatives of E64 include such compounds as those in which the group $-NH-(CH_2)_4-NH-C(=NH)-NH_2$ is replaced by various alkyl groups or by such groups as $-NH-(CH_2)_4-NH_2$, $-NH-(CH_2)_2-CH_3$, $-NH-(CH_2)_7-NH_2$ or $-O_2CCH=CH-CO-NHCH(i\text{-propyl})-CO-NH-(CH_2)_2-CH-(CH_3)_2$.

Preferred cystatin type inhibitors are selected from the group consisting of egg white cystatin, human cystatin A, human cystatin B, human cystatin C, human cystatin S, rat cystatin α, rat cystatin β and kininogen, e.g. L-kininogen and H-kininogen.

A particularly preferred thiolproteinase inhibitor is egg white cystatin.

In addition, transgenic plants may show resistance to pests when in vivo synthesis produces an inhibitor of a metalloproteinase. Preferred inhibitors are those which inhibit carboxypeptidase A, carboxypeptidase B, aminopeptidase, collagenase, calcium-dependent neutral proteinase, thermolysin, angiotensin-converting enzyme, renal dipeptidase, enkephalinase, gelatinase or keratinase or inhibitors of proteinases which have substantial structural or functional similarity to any to these.

Particularly preferred is an inhibitor which is, or has substantial structural or functional similarity to, a potato carboxypeptidase inhibitor, mammalian collagenase inhibitor, α_2-macroglobulin, tissue bradykinin-potentiating peptide, phosphoramidon, bestatin or amastatin.

Finally, inhibition is observed when the inhibitor is an inhibitor of an acidic proteinase. Preferred inhibitors are those which inhibit pepsin, rennin, cathepsin D, chymosin, Penicillinopepsin or *Scytalidium* acid protease B, or inhibitors of proteinases which have substantial structural or functional similarity to any of these.

Particularly preferred as such an inhibitor is one which is, or has substantial structural or functional similarity to, pepstatin, *Ascaris* carboxyl proteinase inhibitor, *Bauhinia* pepsin inhibitor, *Scopolia japonica* proteinase inhibitor or potato cathepsin D inhibitor.

While typical pests of these preferred plants are of insect, acarid, fungal or bacterial origin, insect pests are of special significance. Table B provides some preferred examples of target plants genetically manipulated to produce the indicated proteinase inhibitor(s) which impart(s) control to the indicated target insect. These results are intended to be no way limiting of the invention.

Primary target pests on monocots, for example maize, are members of the order *Coleoptera* or *Lepidoptera*, in particular of the genera *Diabrotica*, *Diatraea*, *Ostrinia* and *Heliothis*, for example corn root worm or corn

borer. Primary target pests on dicots, for example potato, rape, tomato, soybean, pea or cotton are also members of the order *Coleoptera* or *Lepidoptera*, in particular of the genera *Diabrotica*, *Diatraea*, *Ostrinia*, *Heliothis*, *Spodoptera* and *Anthonomus*, for example potato beetle.

The proteinase inhibitor may be expressed in any part of the plant, for example in the roots, stalks, leaves, seed or pollen of the plant. Preferably the proteinase inhibitor is expressed in that plant part which is the primary attack point of the pest to be controlled.

(2) Transgenic Plants Containing, and Use of, Inhibitors of Non-trypsin Proteinase

The present invention also provides a means of controlling pests on an extensive number of transgenic target monocot and dicot plants using proteinase inhibitors which are representative of all four conventional classes and which are biologically synthesized in those plants. More specifically the invention relates to using inhibitors of non-trypsin proteinases of all four classes, preferably to using inhibitors of non-trypsin serine proteinases. Pests that can be controlled in this manner are predominantly of insect, acarid, fungal or bacterial origin.

Plants of particular interest to this aspect of the present invention include monocots and dicots in Table A as mentioned above selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage and Turf Grasses, Forage Legumes, Fiber Plants and Woods, and Spices and Flavorings, and further to these plant types also Drug Crops.

Preferred monocot plant types include those of the Cereals, Vegetables and Tubers, Sugar Crops and Forage and Turf Grasses. Particularly preferred are plants of the genera *Avena* (oats), *Hordeum* (barleys), *Oryza* (rice), *Sorghum* (sorghum), *Triticum* (wheats), *Dactylis* (cocksfoot, orchard grass) and *Saccharum* (sugar cane), and *Zea mays* (maize). Most preferred are plants of the genus *Dactylis* and *Zea mays*.

Preferred dicot plant types include those of the Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Drug Crops. Particularly preferred are plants of the genera *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales), *Gossypium* (cottons) and *Nicotiana* (tobaccos). Most preferred are plants of the genera *Lycopersicon*, *Solanum*, *Gossypium* and *Nicotiana*.

Inhibition may be observed in transgenic plants containing a foreign gene or gene group encoding an inhibitor of a non-trypsin serine proteinase. Preferably that is an inhibitor of at least one non-trypsin serine proteinase selected from the group consisting of thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, $C\bar{1}$-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent protease, thermitase, mast cell proteinase I and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio* α-proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, or of a proteinase which has substantial structural or functional similarity to any of these. In addition, the inhibitor may be an inhibitor of chymotrypsin. When the proteinase is chymotrypsin, the inhibitor is preferably a potato I, potato II, tomato I or tomato II inhibitor.

Inhibition is also observed as a result of in vivo synthesis of other inhibitors of non-trypsin serine proteinases when one or more of such inhibitors is a member of the Bowman-Birk inhibitor family, the *Streptomyces* subtilisin inhibitor family, the potato inhibitor I family, the potato inhibitor II family, the α_1-proteinase inhibitor family, the hirudin family, the bdellin family, the eglin family, the inter-α_1 trypsin inhibitor family, the serpin superfamily, the $C\bar{1}$-inhibitor family, the *Ascaris* inhibitor family, the leupeptins, the antipains, elastinal and chymostatin, or an inhibitor which has substantial structural of functional similarity to any of these.

Particularly preferred non-trypsin serine protease inhibitors of this aspect of the invention are eglin C and eglin C mutants, especially eglin C (Arg 45).

A preferred group of inhibitors biologically synthesized in the transgenic plants of this aspect of the invention and useful in controlling pests, particularly insect pests, contains an inhibitor of a thiol-proteinase mentioned above. Preferred and particularly preferred examples of such inhibitors are listed in the preceding sections and are also preferred in this aspect of the invention.

In addition, transgenic plants may show resistance to pests when in vivo synthesis produces an inhibitor of a metalloproteinase. Preferred and particularly preferred inhibitors of this aspect of the invention are those which are mentioned as preferred in the preceding sections.

Finally, inhibition is observed when the inhibitor is an inhibitor of an acidic proteinase. Again preferred and particularly preferred inhibitors of this aspect of the invention are those which are mentioned as preferred in the preceding sections.

While typical pests of these preferred plants are of insect, acarid, fungal or bacterial origin, insect pests are of special significance. Table B provides some preferred examples of target plants genetically manipulated to produce the indicated proteinase inhibitor(s) which impart(s) control to the indicated target insect. These results are intended to be no way limiting of the invention. Typical pests to be treated are mentioned in the preceding sections.

D. DNA Sequences

In order to carry out the transformation of plants that is needed to generate the transgenic plants of this invention and to practice the method of this invention, DNA sequences which code for the proteinase inhibitors, or for precursors of the proteinase inhibitors, must be

available for manipulation. Accordingly this invention also embraces the DNA sequences so utilized. Techniques for carrying out these transformations are described in detail subsequently.

Generically, the invention is directed to a substantially pure DNA sequence which comprises a coding sequence for a proteinase inhibitor selected from the group consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases, or a coding sequence for one or more precursors participating in the biological synthesis of a proteinase inhibitor, preferably a non-trypsin proteinase inhibitor, selected from the group consisting of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases. In addition, the invention is directed to a substantially pure DNA sequence coding for a proteinase inhibitor selected from the group consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases.

The invention is also intended to embrace vectors which carry sequences encoding proteinase inhibitors, or precursors of proteinase inhibitors, and such sequences isolated in substantially pure form.

Preferred are those sequences coding for inhibitors which are effective against insect, acarid, fungal or bacterial pests. Of particular importance are those sequences coding for inhibitors, or precursors of inhibitors, which are effective against insects.

Typically the sequence will be one wherein the inhibitor gene originates from, or has substantial sequence homology to a proteinase inhibitor gene originating from, an animal, a bacterium or a fungus or from a plant of a species different from that of the target plant.

When the invention is directed to an inhibitor of a non-trypsin serine proteinase, it may be selected from the group consisting of inhibitors of chymotrypsin, thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, $C\bar{1}$-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent protease, thermitase, mast cell proteinase I

and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio* α-proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

As mentioned previously, the invention embraces DNA sequences which encode one or more precursors participating in the biological synthesis of a proteinase inhibitor wherein said inhibitor is selected from the group consisting of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases. In this instance the serine proteinase is selected from the group consisting of trypsin, chymotrypsin, thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, $C\bar{1}$-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent protease, thermitase, mast cell proteinase I and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio* α-proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, and a proteinase which has substantial structural or functional similarity to any of these.

More specifically, the sequence(s) may be one or more which code for precursors leading to an inhibitor that is a member of the Bowman-Birk inhibitor family, the soybean Kunitz inhibitor family, the bovine pancreatic trypsin (Kunitz) inhibitor family, the Kazal trypsin inhibitor family, the *Streptomyces* subtilisin inhibitor family, the potato inhibitor I family, the potato inhibitor II family, the α_1-proteinase inhibitor family, the hirudin family, the bdellin family, the eglin family, the inter-α_1 trypsin inhibitor family, the serpin superfamily, the $C\bar{1}$-inhibitor family, the *Ascaris* inhibitor family, the leupeptins, the antipains, elastinal and chymostatin, or an inhibitor of chymotrypsin or an inhibitor having substantial structural or functional similarity to an inhibitor of chymotrypsin. In this last case the sequence may be one encoding an inhibitor wherein this inhibitor is a potato I, potato II, tomato I or tomato II inhibitor.

In another embodiment the DNA sequence may encode a proteinase inhibitor, or one or more precursors of a proteinase inhibitor, belonging to the class of inhibitors of thiolproteinases. In this instance the inhibitor may be an inhibitor of papain, bromelain, ficin, calpain, cathepsin B, cathepsin C, cathepsin L, cathepsin H, cathepsin S, chymopapain, clostripain, asclepain, prolyl endopeptidase, pyroglutamyl peptidase, dipeptidyl proteinase I, yeast proteinase B, *Streptococcus* proteinase, *Staphylococcus* thiol proteinase or actinidin, or an inhibitor which has substantial structural or functional similarity to any of these.

Particularly preferred in this situation is an inhibitor which is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64 or a derivative thereof. If the inhibitor is a cystatin, it may be selected from the group consisting of egg white cystatin, human cystatin A, human cystatin B, human cystatin C, human cystatin S, rat cystatin α, rat cystatin β and kininogen, e.g. L-kininogen and H-kininogen.

Furthermore, the sequence may encode a proteinase inhibitor, or one or more precursors of a proteinase inhibitor, which is an inhibitor of a metalloproteinase. For example, the inhibitor may be an inhibitor of carboxypeptidase A, carboxypeptidase B, aminopeptidase, collagenase, calcium-dependent neutral proteinase, thermolysin, angiotensin-converting enzyme, renal dipeptidase, enkephalinase, gelatinase or keratinase, or an inhibitor of a proteinase having substantial structural or functional similarity to any of these.

Particularly preferred in this instance are DNA sequences which encode inhibitors, or one or more precursors of inhibitors, wherein the inhibitors are, or have substantial structural or functional similarity to, a potato carboxypeptidase inhibitor, mammalian collagenase inhibitor, α_2-macroglobulin, tissue bradykinin-potentiating peptide, phosphoramidon, bestatin or amastatin.

Finally, the invention embraces DNA sequences encoding a proteinase inhibitor, or one or more precursors of a proteinase inhibitor, which is an inhibitor of an acidic proteinase. Of particular interest are those

cases wherein the inhibitor is an inhibitor of pepsin, rennin, cathepsin D, chymosin, penicillinopepsin or *Scytalidium* acid protease B, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

Particularly preferred are those situations wherein the inhibitor is, or has substantial structural or functional similarity to, pepstatin, *Ascaris* carboxyl proteinase inhibitor, *Bauhinia* pepsin inhibitor, *Scopolia japonica* proteinase inhibitor or potato cathepsin D inhibitor.

E. Vectors

Vectors, produced by standard techniques and comprising the DNA sequences described above, represent an additional feature of the invention. Vectors are recombinant DNA sequences which may be used for isolation and multiplication purposes of the mentioned DNA sequences and for the transformation of suitable hosts with these sequences. Preferred vectors for isolation and multiplication are plasmids which can be propagated in a suitable host microorganism, for example in *E. coli*. Preferred vectors for transformation are those useful for transformation of plant cells or of *Agrobacterium*. More particularly, if plant cells other than protoplasts are being transformed, then the preferred vector is a Ti-plasmid derived vector. For the direct DNA transfer into protoplasts, any of the mentioned vectors may be used. Appropriate vectors which can be utilized as starting materials are known in the art. Suitable vectors for transforming plant tissue and protoplasts have been described by deFramond et al. (1983); An et al. (1985); Potrykus et al. (1985); Rothstein et al. (1987). In addition to these, many other vectors have been described in the art which are suitable for use as starting materials in the present invention.

The construction and multiplication of the vectors can be performed in a suitable host, for example, in *E. coli*. Suitable *E. coli* strains include HB101, JM83, DH1, DH5α, LE392 and the like. The vectors of the invention may be used as such in a direct gene transfer or a micro-injection technique. In certain instances it may be preferable to linearize the vector before use. Alternatively the vectors may be transferred to an

Agrobacterium host. This transfer is accomplished by conventional techniques including biparental mating (Simon et al., 1983b), triparental mating (Ditta et al., 1980) or transformation (Holsters et al.,1978). Suitable strains of *Agrobacterium* include but are not limited to *A. tumefaciens* LBA4404, CIB542 and C58Z707.

Preferred vectors are those comprising the preferred DNA sequences mentioned above. Furthermore a preferred vector is one that is functional in plant cells or in *Agrobacterium*. Particularly preferred are the vectors described in the Examples.

F. Processes of Preparation

The invention further embraces a process for the preparation of the mentioned DNA sequences wherein the DNA sequence is isolated from a naturally occurring source, optionally mutated, or synthesized chemically or enzymatically.

The invention also embraces a process for the preparation of the mentioned vectors comprising a DNA sequence as defined above wherein said DNA sequence is ligated into a vector functional in plant cells or in *Agrobacterium*.

The invention further embraces a process for the preparation of transgenic plants as defined above wherein a plant cell is transformed with a vector comprising a DNA sequence of the invention or is co-cultivated with *Agrobacterium* containing such a vector, and the plant cell is regenerated into a plant.

Preferred processes are those which lead to the preferred DNA sequences, vectors and transgenic plants of the invention. Particularly preferred are the processes described in the Examples.

EXAMPLES

The following examples are provided in order to demonstrate both general and specific aspects of the invention. Accordingly, they are not intended to limit the scope of the disclosure or claims in any way.

ABBREVIATIONS

bp: base pair.

2,4-D: 2,4-dichlorophenoxyacetic acid.

Dicamba: 3,6-dichloro-2-methoxy benzoic acid.

EDTA: 1-ethylendiamine N,N,N',N'-tetraacetic acid

kb: kilo base pair.

MES: 2-(N-morpholino)ethane sulfonic acid.

MW: molecular weight.

NAA: α-naphthaleneacetic acid.

PEG: polyethylene glycol.

rpm: rounds per minute

SDS: sodium dodecyl sulfate.

Tris-HCl: tris(hydroxymethyl)methylamine hydrochloride.

MEDIA

SH-O medium: Medium of Schenk and Hildebrandt (1972); without hormones. SH medium can be liquid or solidified with 0.8 % agar or with 0.5 % GelRite®. The medium is normally sterilized by heat in an autoclave at 110° to 121°C for 15-20 min.

SH-30 medium: SH-O medium containing 30 µM Dicamba.

SH-45 medium: SH-O medium containing 45 µM Dicamba.

MS Medium and OMS medium: Media of Murashige and Skoog (1962). The media can be solidified with 0.8 % agar or agarose or with 0.5 % GelRite®.

Beasley and Ting Medium (embryo germination medium): Medium of Beasley and Ting (1973).

KM-8p Medium: This medium comprises macroelements, microelements and Fe-EDTA as described by Kao and Michayluk (1975), and the following organic compounds: Biotin (0.01 mg/liter), pyridoxine-HCl (1 mg/liter), thiamine-HCl (10 mg/liter), nicotinamide (1 mg/liter), nicotinic acid (0.1 mg/liter), folic acid (0.4 mg/liter), D-calcium-pantothenate (1 mg/liter), p-aminobenzoic acid (0.02 mg/liter), choline chloride (1 mg/liter), riboflavine (0.2 mg/liter), vitamin B12 (0.02 mg/liter), glycine (0.1 mg/liter), sucrose (0.25 g/liter), glucose (68.4 g/liter), mannitol (0.25 g/liter), sorbitol (0.25 g/liter), cellobiose (0.25 g/liter), fructose (0.25 g/liter), mannose (0.25 g/liter), rhamnose (0.25 g/liter), ribose (0.25 g/liter), xylose (0.25 g/liter), myo-inositol (0.1 g/liter), citric acid (40 mg/liter), fumaric acid (40 mg/liter), malic acid (40 mg/liter), sodium pyruvate (20 mg/liter), adenine (0.1 mg/liter), guanine (0.03 mg/liter), thymidine (0.03 mg/liter), uracil (0.03 mg/liter), hypoxanthine (0.03 mg/liter), cytosine (0.03 mg/liter), glutamine (5.6 mg/liter), alanine (0.6 mg/liter), glutamic acid (0.6 mg/liter), cysteine (0.2 mg/liter), asparagine, aspartic acid, cystine, histidine, isoleucine, leucine,

lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine (each 0.1 mg/liter). The solution is filter sterilized. The final pH is 5.8.

Macroelements are made up as a 10 X concentrated stock solution, and microelements as a 1000 X concentrated stock solution. Citric, fumaric and malic acid and sodium pyruvate are prepared as a 100 X concentrated stock solution adjusted to pH 6.5 with NH_4OH. Adenine, guanine, thymidine, uracil, hypoxanthine and cytosine are prepared as a 1000 X concentrated stock solution, adjusted to pH 6.5 with NH_4OH. The amino acids are added using a 10 X stock solution (pH 6.5 with NH_4OH) to yield the given final concentrations. Vitamin stock solution is normally prepared 100 X concentrated.

N6 Medium: This medium comprises macroelements, microelements and Fe-EDTA as described by Chu et al. (1975), and the following organic compounds: Pyridoxine-HCl (0.5 mg/liter), thiamine-HCl (0.1 mg/liter), nicotinic acid (0.5 mg/liter), glycine (2.0 mg/liter), and sucrose (30.0 g/liter). The solution is autoclaved. The final pH is 5.6.
Macroelements are made up as a 10 X concentrated stock solution, and microelements as a 1000 X concentrated stock solution. Vitamin stock solution is normally prepared 100 X concentrated.

YEB Medium: 5 g/liter beef extract, 1 g/liter yeast extract, 5 g/liter peptone, 5 g/liter sucrose, adjusted to pH 7.2 with NaOH, 2 mM $MgCl_2$ added after autoclaving.

Agarose: Preparation and purification of agarose are described, for example by Guiseley and Renn (1975). Agarose is one of the constituents of agar. Commercially available agar normally consists of a mixture of neutral agarose and ionic agaropectin with a large number of side groups. Usually a certain number of side chains remains intact and determines the physicochemical properties of the agarose such as gel formation and melting temperature. Low-melting agarose, especially SeaPlaque® agarose is a preferred solidifying agent within the process described hereinafter.

Casein hydrolysate: Casein Hydrolysate - Enzymatic Hydrolysate from bovine milk, Type 1, Sigma Co., St. Louis, MO, USA.

Cellulase RS and R-10: Yakult Honsha Co. Ltd., Tokyo, Japan.

GelRite®: GelRite Gellan Gum, Scott Laboratories Inc., Fiskersville, R.I., USA.

Nalgene®filter: Nalge Co., Division of Sybron Corp., Rochester, New York, USA.

Pectolyase Y-23®: Seishin Pharmaceutical Co. Ltd., Tokyo, Japan.

Parafilm®: Parafilm® laboratory film - American Can Co., Greenwich, CT, USA.

GENERAL RECOMBINANT DNA TECHNIQUES

Since many of the recombinant DNA techniques used in this invention are routine for those skilled in the art, a brief description of these commonly used techniques is included here rather than at each instance where they appear below. Except where noted, all of these routine procedures are described in the reference by Maniatis et al. (1982).

A. Restriction Endonuclease Digestions. Typically, DNA is present in the reaction mixture at approximately 50 to 500 µg/ml in the buffer solution recommended by the manufacturer, New England Biolabs, Beverly, MA. 2 to 5 units of restriction endonucleases are added for each µg of DNA, and the reaction mixture incubated at the temperature recommended by the manufacturer for one to three hours. The reaction is terminated by heating to 65°C for ten minutes or by extraction with phenol, followed by precipitation of the DNA with ethanol. This technique is also described on pages 104-106 of the Maniatis et al. reference.

B. Treatment of DNA with Polymerase to Create Flush Ends. DNA fragments are added to a reaction mixture at 50 to 500 µg/ml in the buffer recommended by the manufacturer, New England Biolabs. The reaction

mixture contains all four deoxynucleotide triphosphates at a concentration of 0.2 mM. The reaction is incubated at 15°C for 30 minutes, and then terminated by heating to 65°C for ten minutes. For fragments produced by digestion with restriction endonucleases that create 5'-protruding ends, such as EcoRI and BamHI, the large fragment, or Klenow fragment, of DNA polymerase is used. For fragments produced by endonucleases that produce 3'-protruding ends, such as PstI and SacI, T4 DNA polymerase is used. Use of these two enzymes is described on pages 113-121 of the Maniatis et al. reference.

C. Agarose Gel Electrophoresis and Purification of DNA Fragments from Gels. Agarose gel electrophoresis is performed in a horizontal apparatus as described on pages 150-163 of the Maniatis et al. reference. The buffer used is the Tris-borate buffer described therein. DNA fragments are visualized by staining with 0.5 µg/ml ethidium bromide, which is either present in the gel and tank buffer during electrophoresis or added following electrophoresis. DNA is visualized by illumination with short-wavelength or long-wavelength ultraviolet light. When the fragments are to be isolated from the gel, the agarose used is the low temperature gelling variety, obtained from Sigma Chemical, St. Louis, Missouri. After electrophoresis, the desired fragment is excised, placed in a plastic tube, heated to 65°C for approximately 15 minutes, then extracted with phenol three times and precipitated with ethanol twice. This procedure is slightly modified from that described in the Maniatis et al. reference at page 170.

D. Addition of Synthetic Linker Fragments to DNA Ends. When it is desired to add a new restriction endonuclease site to the end of a DNA molecule, that molecule is first treated with DNA polymerase to create flush ends, if necessary, as described in section B above. Approximately 0.1 to 1.0 µg of this fragment is added to approximately 100 ng of phosphorylated linker DNA, obtained from New England Biolabs, in a volume of 20 to 30 µl containing 2 µl of T4 DNA ligase, from New England Biolabs, and 1 mM ATP in the buffer recommended by the manufacturer. After incubation overnight at 15°C, the reaction is terminated by heating the mixture at 65°C for ten minutes. The reaction mixture is then diluted to approximately 100 µl in a buffer suitable for the restriction endo-

nuclease that cleaves at the synthetic linker sequence, and approximately 50 to 200 units of this endonuclease are added. The mixture is incubated at the appropriate temperature for 2 to 6 hours, then the fragment is subjected to agarose gel electrophoresis and the fragment purified as described in C above. The resulting fragment will now have ends with termini produced by digestion with the restriction endonuclease. These termini are usually cohesive, so that the resulting fragment is now easily ligated to other fragments having the same cohesive termini.

E. Removal of 5'-Terminal Phosphates from DNA Fragments. During plasmid cloning steps, treatment of the vector plasmid with phosphatase reduces recircularization of the vector (discussed on page 13 of the Maniatis et al. reference). After digestion of the DNA with the appropriate restriction endonuclease, one unit of calf intestine alkaline phosphatase, obtained from Boehringer-Mannheim, Indianapolis, IN, is added. The DNA is incubated at 37°C for one hour, then extracted twice with phenol and precipitated with ethanol.

F. Ligation of DNA Fragments. When fragments having complementary cohesive termini are to be joined, approximately 100 ng of each fragment are incubated in a reaction mixture of 20 to 40 µl containing approximately 0.2 units of T4 DNA ligase from New England Biolabs in the buffer recommended by the manufacturer. The incubation is conducted for 1 to 20 hours at 15°C. When DNA fragments having flush ends are to be joined, they are incubated as above, except the amount of T4 DNA ligase is increased to 2 to 4 units.

G. Transformation of DNA into *E. coli*. *E. coli* strain HB101 is used for most experiments. DNA is introduced into *E. coli* using the calcium chloride procedure described by Maniatis et al. on pages 250-251. Transformed bacteria are selectively able to grow on medium containing appropriate antibiotics. This selective ability allows the desired bacteria to be distinguished from host bacteria not receiving transforming DNA. Determining what antibiotic is appropriate is routine, given knowledge of the drug resistance genes present on incoming transforming DNA and the drug sensitivity of the host bacteria. For example, where the

host bacterium is known to be sensitive to ampicillin and there is a functional drug resistance gene for ampicillin on the incoming transforming DNA, ampicillin is an appropriate antibiotic for selection of transformants.

H. Screening *E. coli* for Plasmids. Following transformation, the resulting colonies of *E. coli* are screened for the presence of the desired plasmid by a quick plasmid isolation procedure. Two convenient procedures are described on pages 366-369 of the Maniatis et al. reference.

I. Large Scale Isolation of Plasmid DNA. Procedures for isolating large amounts of plasmids in *E. coli* are found on pages 88-94 of the Maniatis et al. reference.

J. Cloning into M13 Phage Vectors. In the following description, it is understood that the double-stranded replicative form of the phage M13 derivatives is used for routine procedures such as restriction endonuclease digestions, ligations, etc.

I. Identification of Proteinase Inhibitors of Interest

Example 1: In Vitro Assay for Selection of Protease Inhibitor(s) Effective against Target Insects

A. Assay Procedure. Protease Inhibitors are initially screened for activity against the desired target insect by measuring the ability of particular inhibitors to inhibit proteolysis by homogenates of the insect gut. Guts are dissected from CO_2-anesthetized or frozen second or third instar larvae; guts are frozen on dry ice immediately after dissection. Guts are homogenized in 100 mM Tris, 10 mM EDTA, pH 8.5 (Wolfson and Murdock, 1987) using 5 to 10 µl buffer per gut. The homogenate is centrifuged at 5000 rpm for 5 min at 4°C to remove particulates and the supernatant is stored in 1 ml aliquots at -20°C. Protease activity is measured essentially using the method of Wolfson and Murdock (1987) using ^{14}C-BSA (New England Nuclear) as the substrate and measuring the rate of

solubilization of radioactivity with time over a 32 min time period. The pH optimum for protease activity is determined by measuring the amount of hydrolysis in each of the following buffers:

pH	Buffer
2.0	200 mM glycine/HCl
3.0	200 mM glycine/HCl
4.0	200 mM β-alanine/HCl
5.0	200 mM sodium acetate
6.0	100 mM sodium phosphate/biphosphate
7.0	100 mM sodium phosphate/biphosphate
8.0	100 mM Tris/HCl
9.0	200 mM glycine/NaOH

Subsequent experiments are performed at the pH of maximum activity. Inhibitor is incubated with the homogenate and assay buffer for 5 min at 25°C. The substrate, ^{14}C-methylated bovine serum albumin (NEN Products, Boston) adjusted to a specific activity of ~ 0.5 µCi/mg with unlabelled BSA, is added and 20 µl aliquots are removed at 0, 2, 4, 8, 16 and 32 min. These aliquots are added to 200 µl ice-cold 10 % trichloroacetic acid solution and stored on ice for 30 min. Samples are centrifuged for 10 min at 5000 rpm at 4°C to precipitate insoluble protein. Two 75 µl aliquots of each supernatant are added to 8 to 10 ml of Scintiverse II scintillation cocktail to count the radioactive decay events. The rate of solubilization of radioactivity is determined and the rate for each inhibitor is compared with that of the control.

B. Screening with Gut Homogenates from Southern Corn Rootworm *(Diabrotica undecimpunctata)*. In a typical experiment using gut homogenates from *Diabrotica undecimpunctata*, the pH of optimum hydrolysis is found to be pH 4. A number of inhibitors are tested in a standard assay reaction containing 10 µl homogenate, 80,000 cpm ^{14}C-BSA in a final volume of 200 µl 0.2 M β-alanine HCl, pH 4.0. Pepstatin activity is assayed in 0.2 M glycine HCl, pH 2.0. Typical data are presented in Table 1.

Table 1: Effect of Proteinase Inhibitors on Proteinase Activity of Homogenates of *D. undecimpunctata* Guts

Class	Inhibitor	Conc. (µg/µl)	% of Control
Thiol	E-64	0.25	7.2
	Antipain	0.1	19.8
	Cystatin	0.072	30.9
Thiol + Serine	Leupeptin	0.1	21.6
Serine	Aprotinin (Bovine pancreatic trypsin inhibitor)	0.1	85.4
	Chick trypsin inh.	0.1	62
	Potato I chymotrypsin inhibitor	0.1	55.4
	Potato II chymotrypsin inhibitor	0.1	42.8
	Soybean I (Kunitz) trypsin inhibitor	0.1	69.7
	Soybean II (Bowman-Birk)	0.1	72.5
	Lima bean trypsin inhibitor	0.1	161
Acidic	Pepstatin	0.02	2.1

Proteolysis by gut homogenates of *D. virgifera* and *D. balteata* show similar strong inhibition by inhibitors of thiol proteases.

C. Inhibition of Proteolysis in Gut Homogenates from Western Corn Root Worm *(Diabrotica virgifera)* and European Corn Borer *(Ostrinia nubilalis)*. Proteinase inhibitors are tested for their ability to inhibit proteolytic activity in homogenates of guts from both *Diabrotica virgifera* and *Ostrinia nubilalis* using the methods described above. The results shown in Table 2 below are consistent with data obtained with other inhibitors which show that gut proteases in *Ostrinia* are inhibited by serine protease inhibitors while those in *Diabrotica* are not. In particular eglin C (Arg45) is shown to be highly effective in inhibiting proteolysis by *O. nubilalis* gut homogenates; eglin C (Arg45) is significantly more effective than eglin C and another trypsin inhibitor, cowpea trypsin inhibitor (Hilder et al., 1987).

Table 2: Inhibition of Proteolysis in Insect Gut Homogenates

Inhibitor	Conc. (µg/µl)	Percent of control activity	
		D. virgifera	*O. nubilalis*
Eglin C	0.5	107.6 %	29.9 %
Eglin C (Arg45)	0.5	110.8 %	3.4 %
Leupeptin	0.002	6.2 %	24.9 %
Cowpea trypsin inhibitor	0.5	99.2 %	29.5 %

Table 3: Concentration of Inhibitors Required for 70 % Inhibition of Proteolysis by *O. nubilalis* Gut Homogenates

Inhibitor	IC70
Eglin C (Arg45)	12 µM
Eglin C	62 µM
Cowpea trypsin inhibitor	50 µM

Example 2: Feeding Assay for Selection of Protease Inhibitors Effective against Target Insects

An alternative method of determining the efficacy of inhibitors against a particular target insect is to incorporate the inhibitor into the diet. Inhibitor solution is applied to the surface of the normal diet (e.g. young corn leaves for *Ostrinia nubilalis* and *Heliothis zea*, tobacco leaf disks for *H. virescens*, corn roots or cultured corn tissue for *Diabrotica* sp.) and allowed to dry. Newly hatched larvae are exposed to this diet and the survival rate and weight measured after 5 to 6 days. Alternatively, second or third instar larvae may be weighed before and after three days exposure to the treated diet.

A. Effect of Proteinase Inhibitors of Growth of *Heliothis zea*. In a typical experiment corn leaf strips (1 x 1 cm) are coated with 12 µl aliquots of solutions of 10 mg/ml test material and placed in individual diet cups with one newly hatched *H. zea* larva. The weight of the larvae is measured after 5 days. Control proteins such as bovine serum albumin or thaumatin cause a significant ($P<0.05$) increase in weight compared to untreated leaves. Leaves with serine protease inhibitors such as Kunitz soybean trypsin inhibitor or lima bean trypsin inhibitor cause a significant decrease in larval weight compared to the control (Table 4).

Table 4: Effect of Proteinase Inhibitions on Growth of Newly Hatched *H. zea*

Treatment	Mean wt (mg)	n	p
untreated	3.41	15	
BSA	6.65	13	0.005
thaumatin	4.57	15	0.1
soybean trypsin inhibitor I	1.59	19	0.005
lima bean trypsin inhibitor	2.38	13	0.1

B. Effect of Proteinase Inhibitors on Growth of *Diabrotica*. Feeding assays with *Diabrotica* sp. corroborate the in vitro assay described in Example 1. In a representative experiment cells from a corn cell suspension culture are soaked in 2.5 % sucrose containing desired amounts of inhibitors and fed to second instar *D. undecimpunctata* larvae. Weight gain after 5 days is significantly (t test $p<0.05$) lower than controls when cells are treated with leupeptin, antipain, or pepstatin at concentrations exceeding 0.3 mg/ml. In similar experiments *D. virgifera* larval growth is inhibited by doses of leupeptin as low as 0.1 mg/ml (Table 5).

Table 5: Effect of Protease Inhibitors on Growth of *Diabrotica* Larvae

Species	Inhibitor	Dose (mg/ml)	Mean wt (mg)	p
D. undecimpunctata	None		7.54	
	Leupeptin	1.0	5.53	0.05
		3.0	5.02	0.05
		10.0	3.55	0.05
	Antipain	1.0	5.44	0.05
		3.0	5.79	0.05
		10.0	3.69	0.05
	Pepstatin	0.1	7.42	NS
		0.3	6.18	0.05
		1.0	5.99	0.05
		3.0	6.2	0.05
D. virgifera	None		3.67	
	Leupeptin	0.1	2.51	0.05
		0.3	1.91	0.05
		1.0	1.56	0.05
		3.0	1.28	0.05

C. Effect of Proteinase Inhibitors on Growth of *Ostrinia*. The effect of eglin C (Arg45) on the growth of newly hatched *Ostrinia nubilalis* larvae is determined using the asssay described above. Aliquots of inhibitor solution are applied to 1 x 1 cm pieces of corn leaf; each leaf piece is placed in a separate diet cup along with one newly hatched larva. Twenty larvae are tested at each dose. The cups are incubated at 29°C for six days after which survival rate, final insect weight and leaf area eaten are scored. Application of 125 µg of eglin C (Arg45) to each leaf piece results in a significant decrease in final weight of the larvae ($p>0.05$). Typically exposure to eglin C (Arg45) results in a decrease of about 30 % in the average final larval weight when compared to untreated controls.

II. Genes and Vectors

A protease inhibitor gene is isolated from the source in which it exists naturally or synthetically and, if necessary, characterized by conventional methods. If the gene is inducable, it is activated by the appropriate regulator. RNA resulting from that activation is isolated and is used to construct a cDNA library. This library is used for differenti-

al screening using radiolabelled cDNA generated from (1) RNA isolated from the activated system and (2) RNA isolated from a second system not so activated. cDNA clones corresponding to induced clones are then isolated and sequenced.

If the desired gene is not known to be inducable, it may be isolated using immunological procedures. The protease inhibitor protein is used as antigen to produce antibody, which is then used to screen a recombinant phage cDNA library in λ-GT11 according to the procedure of Young and Davis (1983). Positive clones are screened with radiolabelled mixed oligonucleotides (Wood et al., 1985) generated according to the known amino acid sequence of the protein.

Example 3: Proteinase Inhibitor Genes

A. Purification of Proteinase Inhibitors. Many proteinase inhibitors are available commercially in purified or partially purified form and the purification protocols for numerous others have been published [see Barrett and Salvesen (1986) for extensive references]. Further purification of material can be effected using affinity chromatography on columns of the appropriate inactivated enzyme.

In a typical purification with pineapple bromelain inhibitor, the enzyme column is prepared with bromelain essentially as described by Anastasi et al. (1983) for carboxymethylpapain. The bromelain is reacted with iodoacetamide to inactive it. The carboxymethyl-bromelain is then coupled to CNBr-activated Sepharose (Pharmacia) using the protocol recommended by the manufacturer. After extensive washing the column is equilibrated in 0.05 M $NaPO_4$, pH 4.0, 0.5 M NaCl, 0.1 % Brij 35 and treated with one column volume of 50 mg/ml bovine serum albumin in the same buffer to block non-specific binding sites. The column is thoroughly washed and the partially purified bromelain (Sigma) dissolved in pH 4.0 buffer containing 10 % glycerol instead of Brij 35 is applied to the column. After thorough washing with pH 4.0 buffer the column is eluted with 0.05 M $NaPO_4$, pH 11.5, 0.5 M NaCl, 10 % glycerol. Activity is monitored by testing for the ability to inhibit the degradation of ^{14}C-BSA by bromelain.

When isolating inhibitors which have a high degree of activity on the protease of a particular insect the efficiency of purification can be tested using the assay described in Example 1. To increase selectivity, the insect enzyme can be purified by affinity chromatography on an inhibitor column and then used to make an affinity column for purification of new inhibitors.

If necessary further purification of inhibitors for protein sequencing can be effected using reverse phase HPLC.

B. Amino Acid Sequencing of Proteinase Inhibitors. In the case of proteins whose amino acid sequence is not already available in the literature, the amino acid sequence is determined by automatic Edman degradation using a Model 470A Protein Sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line reverse phase HPLC for analysis of the phenylthiohydantoin derivatives of the amino acids and a Model 900 data analysis system. Peptides are produced by enzymatic digestion with trypsin, Lys-C (lysine endopeptidase), Arg-C (arginine endopeptidase) or Glu-C (*Staphylococcus aureus* protease V8) and separated by reverse phase HPLC prior to sequencing.

C. Synthesis of a Gene Encoding Proteinase Inhibitor: Chicken Egg White Cystatin. For proteins of less than 150 amino acids whose amino acid sequence is entirely known, a gene may be constructed by DNA synthesis. In the case of chicken egg white cystatin, the amino acid sequence (Schwabe et al., 1984; Turk et al., 1983) is back-translated using the genetic code with the codon frequency calculated from all available corn proteins in the GenBank data bank using the computer programs of the University of Wisconsin Genetics Computer Group. Translational stop and start signals are added along with BamHI linkers at both ends for convenience in subsequent manipulations. This process yields the sequence shown in Figure 9.

Oligonucleotides corresponding to the regions A-K (Figure 10) are synthesized using a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA) with β-cyanoethyl chemistry.

The gene is assembled in three steps:
(1) Addition of 5'-phosphate: A 5'-phosphate is added to the 5' ends of all fragments except fragments A and K by mixing 40 pmol of each fragment B, C, D, E, F, G, H, I and J with T4 polynucleotide kinase according to the method described in Maniatis et al. (1982).

(2) Annealing: After removal of excess reagent by phenol/chloroform extraction, chloroform extraction, and ethanol precipitation, the precipitate containing the phosphorylated fragments is dissolved in T4 ligase buffer. 40 pmol each of fragment A and K are added, the mixture is heated to 85°C, then slowly cooled to 15°C and maintained at 15°C for at least 4 h to allow the fragments to anneal.

(3) Ligation: ATP is added to 1 mM along with T4 ligase and incubation is continued for 4 h. Reagents are removed by extraction and precipitation follows as in step (1). To check for the efficacy of the reaction, an aliquot of the products is analyzed on a 10 to 15 % acrylamide gel. A 363 bp band is visible. If necessary the corresponding DNA fragments may be purified by preparative gel electrophoresis before proceeding to ligate it into the vector.

D. Synthesis of a Gene Encoding a Proteinase Inhibitor: Eglin C and Eglin C Mutants.
(1) Eglin C Gene: The preparation of the plasmid pML147 containing the eglin C gene is described in the European Patent Application 146 785. The strain *E. coli* HB101/pML147 harboring this plasmid was deposited on January 28, 1988 at the Deutsche Sammlung von Mikroorganismen (DSM), Mascheroder Weg 1b, D-3300 Braunschweig, Federal Republic of Germany, under the number DSM 4380. The 230 bp EcoRI-BamHI fragment containing the complete eglin C gene is isolated from 10 µg of plasmid pML147 by means of digestion with the restriction endonucleases EcoRI and BamHI and subsequent electrophoresis on 1.5 % low melting point agarose. The EcoRI end is converted to a BamHI end by ligating with the appropriate linker chosen so that the coding sequence will be in the correct reading frame. After isolation, the modified fragment is inserted into the BamHI site of the CaMV35S promoter in the pCIB710 vector as described in Example 5.

(2) M13 Cloning of the Eglin C Gene: Approximately 0.5 µg of the 230 bp EcoRI-BamHI fragment containing the complete eglin C gene is isolated as above. This DNA fragment (10 ng) is mixed with 40 ng of M13mp8 pre-digested with EcoRI and BamHI and incubated in 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM ATP, 10 mM dithiothreitol in the presence of 0.125 units of T4 DNA ligase in a volume of 15 µl (Zoller et al., 1983). The resulting solution is used to transform the *E. coli* strain JM101 (Zoller et al., 1983). The transformation mixture is coated onto X-Gal (IPTG-indictor agar) plates (Zoller et al., 1983). 40 blue (wild type) plaques and 650 colorless plaques are obtained.

(3) Production of M13mp8 Single-stranded DNA: 2 ml of a culture of *E. coli* JM101 grown in L medium (10 g/liter Bacto tryptone, 5 g/liter Bacto yeast extract, 5 g/liter NaCl, 5 g/liter glucose, 0.1 g/liter ampicillin) up to an OD_{623} of approximately 0.5 are inoculated with a colorless plaque taken from the agar plate (see above) and maintained for approximately 4 to 5 hrs at 37°C and 180 rpm. Subsequently the grown culture is centrifuged for 5 min in a Eppendorf centrifuge. The supernatant is transferred into a fresh centrifuge tube and centrifuged again. 200 µl of 20 % PEG, 1.5 M NaCl are added, and the mixture is maintained at room temperature for 20 min and then centrifuged again. The supernatant is discarded and the pellet is dissolved in 100 µl 50 mM Tris-HCl, pH 7.8, 1 mM EDTA (TE buffer). The mixture is mixed with 50 µl phenol/TE buffer, kept for 15 min at room temperature and then centrifuged for 5 min in a Eppendorf centrifuge. 10 µl sodium acetate buffer, pH 6, and 250 µl absolute ethanol are added to 100 µl of supernatant, and the mixture is maintained at -20°C overnight and then centrifuged as described above for 10 min. The pellet is dried at room temperature for 10 min and then dissolved in 50 µl of TE buffer. The solution contains approximately 5 µg of M13mp8 single-stranded DNA.

(4) Production of the Gene Coding for Eglin C (Arg45): A method known as "site directed mutagenesis" according to Zoller et al., (1983) is used. The following nucleotide is produced by chemical synthesis for the mutagenesis of the eglin C gene:

5'-CT CCT GTT ACT C*GG GAC C-3'

The starred base differs from the corresponding base in the coding strand of the eglin C gene (a T) and will finally cause the mutation from Leu45 to Arg45. 10 µl of the oligonucleotide (1 OD/ml = 500 ng) are kinased in 20 µl of 0.07 M Tris-HCl pH 7.6, 0.01 M $MgCl_2$, 50 mM dithiothreitol with γ-^{32}P ATP and T4 polynucleotide kinase (Boehringer) according to the method of Maniatis et al. (1982) p. 125. The kinased oligonucleotide is dissolved in 10 µl TE buffer (50 ng/µl).

1 µg of the M13mp8 single-stranded DNA and 50 ng of the kinased oligonucleotide primer in 10 µl 50 mM Tris-HCl pH 7.8, 100 mM $MgCl_2$ are maintained at 45°C for 30 min and then at room temperature for 5 min for annealing. 1 µl each of 10 mM dATP, dGTP, dCTP and dTTP, 1 µl T4 DNA-ligase, 2 µl 50 mM dithiothreitol, 1 µl 10 mM ATP, 1 µl gelatine (5 mg/ml), 1 µl of 10 x Klenow buffer (0.66 M Tris-HCl pH 7.6, 50 mM $MgCl_2$, 50 mM dithiothreitol), and 1 µl (2.5 units) DNA polymerase (Klenow fragment) are added. The mixture is maintained at 22°C for 5 min and then at 15°C for 16 hrs and finally separated electrophoretically on 1 % agarose. The resulting circular, double-stranded DNA is made visible with ethidium bromide and isolated from the gel by electroelution (approximately 10 ng in 15 µl TE buffer). 5 µl (approximately 3.5 ng) of the DNA obtained in this manner are transformed into *E. coli* strain JM101 and coated onto X-Gal/IPTG indicator plates (see above). Approximately 100 colorless plaques are obtained.

40 of these plaques are used to inoculate each a 2 ml *E. coli* JM101 culture (see paragraph (3)). After cultivation, the *E. coli* cells are centrifuged off from the supernatants containing phages and single-stranded DNA. The cell pellets already contain the corresponding mutated double-stranded DNA. 50 µl of each of the 40 phage supernatants are filtered over nitrocellulose, washed twice with TE buffer, maintained under vacuum for 2 hrs at 80°C and examined according to Southern (1975) for the presence of the mutated DNA sequence using the oligonucleotide primer as a radioactive probe in hybridization. Twelve phage supernatants potentially containing the eglin C (Arg45) gene are identified. Four of these positive phage supernatants are diluted

approximately $1:10^5$, mixed with *E. coli* strain JM101 and coated onto indicator agar. Phages of three each of the resulting plaques are isolated. The single-stranded DNA is isolated therefrom in the manner described above. These 12 single-stranded DNAs are sequenced in accordance with Sanger (1977 and 1981). All 12 single-stranded DNAs exhibit the desired mutated eglin C sequence. In a minipreparation, the respective mutated double-stranded DNA (eglin C (arg45) gene in plasmid M13mp8) is then prepared from the corresponding *E. coli* cell pellets (see above). By restriction digestion with the endonucleases EcoRI and BamHI, the EcoRI-BamHI insert containing the mutated gene is cut out from the vector, isolated and cloned in the vector pHRi148/EcoRI/BamHI (European Patent Application 146 785). The plasmid pJPG18 resulting therefrom is isolated and transformed into *E. coli* strain HB101.

The 230 bp EcoRI-BamHI fragment containing the mutated eglin C (arg45) gene is again cut out of the vector pJPG18 and adapted as described for the non-mutated eglin C gene in paragraph (1) above.

(5) Production of the Gene Coding for Eglin C (Pro44): The Thr44 to Pro44 mutation is carried out in a manner analogous to that descsribed in paragraph (4) above. The mutagenic oligonucleotide used has the following structure:

5'-CT CCT GTT $\overset{*}{C}$CT CTG GAC-3'

The starred base differs from the corresponding base in the coding strand of the eglin C gene (an A) and will finally cause the mutation from Thr44 to Pro44. On working up the mutation mixture 18 potential eglin C (Pro44) mutants are obtained. By cloning the eglin C (Pro44) DNA into the vector pHRi148/EcoRI/BamHI, plasmid pJB591 is obtained and used as above.

Example 4: Construction of a Ti Plasmid-derived Vector.

The vector pCIB10 (Rothstein et al., 1987) is a Ti-plasmid-derived vector useful for transfer of the chimeric gene to plants via *Agrobacterium tumefaciens*. The vector is derived from the broad host range plasmid pRK252, which may be obtained from Dr. W. Barnes, Washington University, St. Louis, Mo. The vector also contains a gene for kanamycin resistance

in *Agrobacterium*, from Tn903, and left and right T-DNA border sequences from the Ti plasmid pTiT37. Between the border sequences are the polylinker region from the plasmid pUC18 and a chimeric gene that confers kanamycin resistance in plants.

First, plasmid pRK252 is modified to replace the gene conferring tetracycline-resistance with one conferring resistance to kanamycin from the transposon Tn903 (Oka et al., 1981), and is also modified by replacing the unique EcoRI site in pRK252 with a BglII site (see Figure 6 for a summary of these modifications). Plasmid pRK252 is first digested with endonucleases SalI and SmaI, then treated with the large fragment of DNA polymerase I to create flush ends, and the large vector fragment purified by agarose gel electrophoresis. Next, plasmid p368 is digested with endonuclease BamHI, treated with the large fragment of DNA polymerase, and an approximately 1050 bp fragment is isolated after agarose gel electrophoresis; this fragment contains the gene from transposon Tn903 which confers resistance to the antibiotic kanamycin (Oka et al., 1981). Plasmid p368 has been deposited with ATCC, accession number 67700. Both fragments are then treated with the large fragment of DNA polymerase to create flush ends. Both fragments are mixed and incubated with T4 DNA ligase overnight at 15°C. After transformation into *E. coli* strain HB101 and selection for kanamycin resistant colonies, plasmid pRK252/Tn903 is obtained.

Plasmid pRK252/Tn903 is digested at its unique EcoRI site, followed by treatment with the large fragment of *E. coli* DNA polymerase to create flush ends. This fragment is added to synthetic BglII restriction site linkers, and incubated overnight with T4 DNA ligase. The resulting DNA is digested with an excess of BglII restriction endonuclease and the larger vector fragment purified by agarose gel electrophoresis. The resulting fragment is again incubated with T4 DNA ligase to recircularize the fragment via its newly added BglII cohesive ends. Following transformation into *E. coli* strain HB101, plasmid pRK252/Tn903/BglII is obtained (see Figure 1).

A derivative of plasmid pBR322 is constructed which contains the Ti plasmid T-DNA borders, the polylinker region of plasmid pUC19, and the selectable gene for kanamycin resistance in plants (see Figure 2). Plasmid pBR325/Eco29 contains the 1.5 kbp EcoRI fragment from the nopaline Ti plasmid pTiT37. This fragment contains the T-DNA left border sequence (Yadav et al., 1982). To replace the EcoRI ends of this fragment with HindIII ends, plasmid pBR325/Eco29 DNA is digested with EcoRI, then incubated with nuclease S1, followed by incubation with the large fragment of DNA polymerase to create flush ends, then mixed with synthetic HindIII linkers and incubated with T4 DNA ligase. The resulting DNA is digested with endonucleases ClaI and an excess of HindIII, and the resulting 1.1 kbp fragment containing the T-DNA left border is purified by gel electrophoresis. Next, the polylinker region of plasmid pUC19 is isolated by digestion of the plasmid DNA with endonucleases EcoRI and HindIII and the smaller fragment (approx. 53 bp) is isolated by agarose gel electrophoresis. Next, plasmid pBR322 is digested with endonucleases EcoRI and ClaI, mixed with the other two isolated fragments, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid, pCIB5, contains the polylinker and T-DNA left border in a derivative of plasmid pBR322 (see Figure 2).

A plasmid containing the gene for expression of kanamycin resistance in plants is constructed (see Figure 4). Plasmid Bin6 is obtained from Dr. M. Bevan, Plant Breeding Institute, Cambridge, UK. This plasmid is described in the reference by Bevan (1984). Plasmid Bin6 DNA is digested with EcoRI and HindIII and the fragment approximately 1.5 kbp in size containing the chimeric neomycin phosphotransferase (NPT) gene is isolated and purified following agarose gel electrophoresis. This fragment is then mixed with plasmid pUC18 DNA which has been cleaved with endonucleases EcoRI and HindIII. Following incubation with T4 DNA ligase, the resulting DNA is transformed into *E. coli* strain HB101. The resulting plasmid is called pUC18/neo. This plasmid DNA contains an unwanted BamHI recognition sequence between the neomycin phosphotransferase gene and the terminator sequence of the nopaline synthase gene (see Bevan, 1984). To remove this recognition sequence, plasmid pUC18/neo is digested with endonuclease BamHI, followed by treatment with the large fragment of DNA polymerase to create flush ends. The fragment is then incubated with T4

DNA ligase to recircularize the fragment, and is transformed into *E. coli* strain HB101. The resulting plasmid, pUC18/neo (Bam) has lost the BamHI recognition sequence.

The T-DNA right border sequence is then added next to the chimeric NPT gene (see Figure 4). Plasmid pBR325/Hind23 contains the 3.4 kbp HindIII fragment of plasmid pTiT37. This fragment contains the right T-DNA border sequence (Bevan et al., 1983). Plasmid pBR325/Hind23 DNA is cleaved with endonucleases SacII and HindIII and a 1.9 kbp fragment containing the right border is isolated and purified following agarose gel electrophoresis. Plasmid pUC18/neo(Bam) DNA is digested with endonucleases SacII and HindIII and the 4.0 kbp vector fragment is isolated by agarose gel electrophoresis. The two fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid, pCIB4 (Figure 4), contains the T-DNA right border and the plant-selectable marker for kanamycin resistance in a derivative of plasmid pUC18.

Next, a plasmid is constructed which contains both the T-DNA left and right borders, with the plant selectable kanamycin-resistance gene and the polylinker of pUC18 between the borders (see Figure 5). Plasmid pCIB4 DNA is digested with endonuclease HindIII, followed by treatment with the large fragment of DNA polymerase to create flush ends, followed by digestion with endonuclease EcoRI. The 2.6 kbp fragment containing the chimeric kanamycin resistance gene and the right border of T-DNA is isolated by agarose gel electrophoresis. Plasmid pCIB5 DNA is digested with endonuclease AatII, treated with T4 DNA polymerase to create flush ends, then cleaved with endonuclease EcoRI. The larger vector fragment is purified by agarose gel electrophoresis, mixed with the pCIB4 fragment, incubated with T4 DNA ligase, and transformed into *E. coli* strain HB101. The resulting plasmid, pCIB2 (Figure 5) is a derivative of plasmid pBR322 containing the desired sequences between the two T-DNA borders.

The following steps complete the construction of the vector pCIB10, and are shown in Figure 6. Plasmid pCIB2 DNA is digested with endonuclease EcoRV, and synthetic linkers containing BglII recognition sites are added as described above. After digestion with an excess of BglII endonuclease,

the approximately 2.6 kbp fragment is isolated after agarose gel electrophoresis. Plasmid pRK252/Tn903/BglII, described above, is digested with endonuclease BglII and then treated with phosphatase to prevent recircularization. These two DNA fragments are mixed, incubated with T4 DNA ligase and transformed into *E. coli* strain HB101. The resulting plasmid is the completed vector, pCIB10.

Example 5: Construction of a chimeric gene with the CaMV 35S promoter

(A) Construction of a CaMV 35S Promoter Cassette Plasmid. pCIB710 is constructed as shown in Figure 7. This plasmid contains CaMV promoter and transcription termination sequences for the 35S RNA transcript (Covey et al., 1981). A 1149 bp BglII restriction fragment of CaMV DNA (bp 6494-7643 in Hohn et al., 1982, 194-220 and Appendices A to G) is isolated from plasmid pLW111 which has been deposited with the American Type Culture Collection on May 14, 1986 and assigned the number ATCC 40235. Alternatively, the fragment can be isolated directly from CaMV DNA by preparative agarose gel electrophoresis. It is mixed with BamHI-cleaved plasmid pUC19 DNA, treated with T4 DNA ligase, and transformed into *E. coli*. (Note the BamHI restriction site in the resulting plasmid has been destroyed by ligation of the BglII cohesive ends to the BamHI cohesive ends.) The resulting plasmid, called pUC19/35S, is then used in oligonucleotide-directed in vitro mutagenesis to insert the BamHI recognition sequence GGATCC immediately following CaMV nucleotide 7483 (as designated in Hohn et al., 1982). The resulting plasmid, pCIB710, contains the CaMV 35S promoter region and transcription termination region separated by a BamHI restriction site. DNA sequences inserted into this BamHI site will be expressed in plants by these CaMV transcription regulation sequences. (Also note that pCIB710 does not contain any ATG translation initiation codons between the start of transcription and the BamHI site.)

(B) Insertion of the CaMV 35S Promoter/Terminator Cassette into pCIB10. The following steps are outlined in Figure 8. Plasmid pCIB10 and pCIB710 DNAs are digested with EcoRI and SalI, mixed and ligated. The resulting plasmid, pCIB10/710 has the CaMV 35S promoter/terminator cassette

inserted into the plant transformation vector pCIB10. The CaMV 35S sequences are between the T-DNA borders in pCIB10, and thus will be inserted into the plant genome in plant transformation experiments.

C. Transfer to *Agrobacterium*. In order to transfer the binary pCIB10 derived plasmid from *E. coli* HB101 to *Agrobacterium*, an intermediate *E. coli* host strain S17-1 is used. This strain, obtainable from Agrigenetics Research Corp., Boulder, Co., is described in Simon et al. (1983a). It contains mobilization functions that can transfer plasmid pCIB10 directly to *Agrobacterium* via conjugation, thus avoiding the necessity to transform naked plasmid DNA directly into *Agrobacterium*. First, plasmid pCIB10 DNA is introduced into calcium chloride-treated S17-1 cells. Next, cultures of transformed S17-1 cells and *A. tumefaciens* strain LBA 4404 (Ooms et al., 1981) are mixed and mated on an N agar (Difco) plate overnight at room temperature. A loopful of the resulting bacteria are streaked onto AB minimal media (Ditta et al., 1980), plated with 50 µg/ml kanamycin and incubated at 28°C. Colonies are restreaked onto the same media, then restreaked onto N agar plates. Slow-growing colonies are picked, restreaked onto AB minimal media with kanamycin and single colonies isolated. This procedure selects for Agrobacteria containing the pCIB10 plasmid.

III. Transformation and Regeneration

Example 6: Transformation of Tobacco

Protoplasts of *Nicotiana tabacum* cv. "Coker 176" are prepared as follows: Four to five week old shoot cultures are grown aseptically in MS medium without hormones at 16°C with a 16 hour light/8 hour dark photoperiod. Approximately 1.5 g of leaf tissue are removed from the plant and distributed equally among 8 to 10 Petri dishes (100 x 25 mm, Lab-Tek), each containing 10 ml of enzyme solution. The enzyme solution contains 1 % cellulase R-10, 0.25 % macerase, from Calbiochem Co., 1 % pectolyase Y-23, from Seishin Pharmaceutical Co., 0.45 M mannitol and 0.1 x K3 salts (Nagy and Maliga, 1976). Tobacco leaves are cut into thin strips with a scalpel, the dishes are sealed, placed on a gyrotory shaker at 35 rpm and incubated with the enzymes for 4 to 5 hours at room temperature.

Next, contents of the dishes are filtered through a cheesecloth-lined funnel and collected in a flask. The filtrate is pipetted into Babcock flasks containing 35 ml each of rinse solution. The rinse solution contains 0.45 M sucrose, 0.1 % MES and 0.1 x K3 salts. The bottles are centrifuged at 80 x g for ten minutes, after which the protoplasts will have floated to the top of the bottle. The protoplasts are removed with a 1 ml pipet, combined into one bottle, and rinsed twice more. The resulting protoplasts are suspended in K_3 medium in a 15 ml disposable centrifuge tube. The concentration of protoplasts is determined by counting in a Fuchs-Rosenthal hemocytometer. The protoplasts are then plated at a density of 100,000/ml in 6 ml of liquid K_3 medium per 100 x 20 mm Petri dish (Corning). The dishes containing the protoplasts are incubated at 26°C in the dark for two days, during which time cell wall regeneration will occur.

After the two-day incubation, 5 µl of a stationary culture of *A. tumefaciens* containing the desired plasmids (grown in YEP medium containing 50 µg kanamycin per ml at 28°C until the stationary phase is reached) are added to the dish of protoplasts. After incubation for three more days at 26°C, cefotaxime (Calbiochem) is added to make a final concentration of 500 µg/ml to kill the Agrobacteria. The following day, cells are diluted with 3 ml fresh K_3 medium per dish, and cefotaxime added again (final concentration 500 µg/ml). Cells are then grown at 26°C for 2 to 3 weeks and then screened on selective medium as described by de Block et al. (1984).

Example 7: Regeneration of Cotton Plants Starting from Cotyledon Explants

A. Cotton Variety Acala SJ2 in Regular Callus Inducing Medium. Seeds of *Gossypium hirsutum* variety Acala SJ2 (cotton) are sterilized by contact with 95 % alcohol for three minutes, then twice rinsed with sterile water and immersed with a 15 % solution of sodium hypochlorite for 15 minutes, then rinsed in sterile water. Sterilized seeds are germinated on a basal agar medium in the dark for approximately 14 days to produce a seedling. The cotyledons of the seedlings are cut into segments which are trans-

ferred aseptically to a callus inducing medium consisting of Murashige and Skoog (MS) major and minor salts supplemented with 0.4 mg/liter thiamine-HCl, 30 g/liter glucose, 2.0 mg/liter NAA, 1 mg/liter kinetin, 100 mg/liter myo-inositol, and agar (0.8 %). The cultures are incubated at about 30°C under conditions of 16 hours light and 8 hours darkness in a Percival incubator with fluorescent lights (cool daylight) providing a light intensity of about 2000 to 4000 lx. Calli are formed on the cultured tissue segments within 3 to 4 weeks and are white to gray-greenish in color. The calli formed are subcultured every three to four weeks onto a callus growth medium comprising MS medium containing 100 mg/liter myo-inositol, 2.0 g/liter sucrose, 2 mg/liter NAA and agar. Somatic embryos form four to six months after first placing the tissue explants on the callus inducing medium. The callus and embryos are maintained on callus growth medium by subculturing onto fresh callus growth medium every three to four weeks.

Somatic embryos which formed on tissue pieces are explanted either to fresh callus growth medium, or to Beasley and Ting (1973)'s medium (embryo germination medium). The somatic plantlets which are formed from somatic embryos are transferred onto Beasley and Ting's medium which contains 15 mg/liter ammonium nitrate and 15 mg/liter casein hydrolysate as an organic nitrogen source. The medium is solidified by a solidifying agent (Gelrite) and plantlets are placed in Magenta boxes. The somatic embryos develop into plantlets within about three months. The plantlets are rooted at the six to eight leaf stage (about 7.5 to 10 cm tall), and are transferred to soil and maintained in an incubator under high humidity for three to four weeks, after which they are transferred to the greenhouse. After hardening, plants are transferred to open tilled soil.

B. Cotton Variety Acala SJ2 in Half-strength Callus Inducing Medium. The procedure of A (above) is repeated using instead half-strength MS medium in which all medium components have been reduced to one-half the specified concentration. Essentially the same results are obtained.

C. Different Cotton Varieties. The procedures of A and B (above) are repeated with Acala cotton varieties SJ4, SJ2C-1, GC510, B1644, B2724, B1810, the picker variety Siokra and the stripper variety FC2017. All are successfully regenerated.

D. Regeneration of Cotton Plants from Cotyledon Explants with Suspension Cell Culture as Intermediate Step. The procedure of A (above) is repeated to the extent of obtaining callus capable of forming somatic embryos. Pieces of about 750 to 1000 mg of actively growing embryogenic callus are suspended in 8 ml units of liquid suspension culture medium comprised of MS major and minor salts, supplemented with 0.4 mg/liter thiamine HCl, 20 g/liter sucrose, 100 mg/liter myo-inositol and NAA (2 mg/liter) in T-tubes and placed on a roller drum rotating at 1.5 rpm under 16 hours light/8 hours dark regime. Light intensity of about 2000 to 4500 lx is again provided by fluorescent lights (cool daylight). After four weeks, the suspension is filtered through an 840 µm size nylon mesh to remove larger cell clumps. The fraction smaller than 840 µm is allowed to settle, washed once with about 20 to 25 ml of fresh suspension culture medium. This cell suspension is transferred to T-tubes (2 ml per tube) and each tube diluted with 6 ml of fresh suspension culture medium. The cultures are maintained by repeating the above at 10 to 12 day intervals. At each subculture, the suspension is filtered and only the fraction containing cell aggregates smaller than 840 µm is transferred to fresh suspension culture medium. In all instances, the fraction containing cell clumps larger than 840 µm is placed onto the callus growth medium to obtain mature somatic embryos. The somatic embryos that are formed on callus growth medium are removed and transferred to embryo germination medium. Using the protocol of A (above), these are germinated, developed into plantlets and then field grown plants.

E. Alternative Intermediate Suspension Culture. The procedure of D (above) is repeated except that suspension cultures are formed by transferring 750 to 1000 mg of embryogenic calli to a DeLong flask containing 15 to 20 ml of liquid MS medium containing 2 mg/liter NAA. The culture containing flask is placed on a gyrotory shaker and shaken at 100 to 110 strokes/minute. After three weeks the suspension is filtered through an 840 µm nylon mesh to remove the large cell clumps for plant

growth, as in D (above). The less than 840 µm suspension is allowed to settle, washed once in the liquid MS medium and resuspended in 2 to 5 ml of liquid MS medium. The suspension is subcultured by transfer to fresh medium in a DeLong flask containing 1 to 2 ml of suspension and 15 ml of fresh liquid MS medium. The cultures are maintained by repeating this procedure at seven to ten day intervals. At each subculture only the less than 840 µm suspension is subcultured and the large clumps (840 µm or greater) are used for plant growth.

F. Production of Plants from Large Clumps of Suspension Cultured Cells. After three or four subcultures using the suspension growth procedure of D and E (above), 1.5 ml to 2.0 ml of cell suspension from the T-tube and DeLong flask are in each instance plated onto agar-solidified MS medium containing 2 mg/liter NAA and Beasley and Ting medium containing 500 mg/liter casein hydrolysate. Within three to four weeks embryogenic calli with developing embryos become visible. Again, the 840 µm or greater cell clumps are plated on the callus growth medium, giving rise to embryogenic clumps with developing embryos, which ultimately grow into plants.

Example 8: Transformation of Cotton Suspension Culture Cells to Tumorous Phenotype by *Agrobacterium* LBA 4434.

A. Growth of the Plant Suspension Culture. An Acala cotton suspension culture is subcultured into "T" tubes with the medium (MS medium containing 2 g/liter NAA) being changed every seven to ten days. After a medium change, the "T" tube is rotated 90° and the cells allowed to settle out. The supernatant is removed by pipeting prior to transformation and the resulting cells treated as described below.

B. Description of the *Agrobacterium* Vector. The *Agrobacterium* strain LBA 4434 (Hoekema et al., 1983) contains a Ti plasmid-derived binary plant transformation system. In such binary systems, one plasmid contains the T-DNA of a Ti-plasmid, the second plasmid contains the vir-region of a Ti-plasmid, and together the two plasmids function to effect plant transformation. In the *Agrobacterium* strain LBA 4434, the T-DNA plasmid pAL1050 contains T_L of pTiAch5, an octopine Ti-plasmid. The vir plasmid

in strain LBA 4434, pAL4404, contains the intact virulence regions of pTiAch5 (Ooms et al., 1982). Strain LBA 4434 is available from Dr. Robert Schilperoort of the Department of Biochemistry, University of Leiden, the Netherlands.

C. Growth of Agrobacteria. The transforming *Agrobacterium* strain is taken from a glycerol stock, inoculated in a small overnight culture, from which a 50 ml culture is inoculated the following day. Agrobacteria are grown on YEB medium to which antibiotics as appropriate have been added. The absorbance at 600 nm of the 50 ml overnight culture is read, the culture is centrifuged and the pellet resuspended in the plant cell growth medium (MS medium containing 2 mg/ml NAA) to a final absorbance at 600 nm of 0.5. 8 ml of this bacterial suspension is added to each "T" tube containing the plant cells from part A above.

D. Infection. The "T" tube containing the plant and bacteria cells is agitated to resuspend all cells and returned to a roller drum for three hours to allow the Agrobacteria to attach to the plant cells. The cells are then allowed to settle and the residual supernatant removed. A fresh aliquot of growth medium is added to the "T" tube and this allowed to incubate on a roller drum for a period of 18 to 20 hours in the presence of any residual Agrobacteria which remained. After this time, the cells are again allowed to settle, the supernatant is removed and the cells are washed twice with a solution of growth medium containing cefotaxime (200 µg/ml). After washing, the cells from each T-tube are resuspended in 10 ml growth medium containing cefotaxime (200 µg/ml in all cases) and 1 ml aliquots of this plated on petri dishes.

E. Growth of Transformed Tissue. The cells infected with Agrobacteria grow on the growth medium which has no added phytohormones, indicating the tissue has received the wild-type phytohormone genes in T-DNA. These cells develop into tumors, further indicating transformation of the cultures.

Example 9: Transformation of Cotton Suspension Culture Cells to a Kanamycin-resistant Non-tumorous Phenotype.

The same procedure as in Example 8 is followed except that different transforming Agrobacteria are used and that the plant selection medium contains an antibiotic for the selection of transformed plant tissue.

A. Growth of Plant Tissue. As in Example 8, part A.

B. Description of the *Agrobacterium* Vector. The transforming Agrobacteria contain the T-DNA containing binary vector pCIB10 (Rothstein et al., 1987) as well as the pAL4404 vir plasmid. The T-DNA of pCIB10 contains a chimeric gene composed of the promoter from the nopaline synthase gene, the coding region from Tn5 (encoding the enzyme neomycin phosphotransferase), and the terminator from the nopaline synthase gene.

C. Growth of Agrobacteria. Agrobacteria containing pCIB10 are grown on YEB containing kanamycin (50 µg/ml). Otherwise, conditions are as in Example 8, part C.

D. Infection. Transformation is accomplished as detailed in Example 8 with the change that the 1 ml aliquots resulting in part C are plated immediately on medium containing selective antibiotics. Selection medium contains either kanamycin (50 µg/ml) or G418 (25 µg/ml). Expression of the nos/neo/nos chimeric gene in transformed plant tissue allows the selection of this tissue on either of these antibiotics.

E. Growth of Transformed Tissue. Plant growth media in this and all following examples contain phytohormones as indicated in Example 7. In 2 to 4 weeks, transformed tissue becomes apparent on the selection plates. Uninfected tissue or control tissue shows no signs of growth, turns brown and dies. Transformed tissue grows very well in the presence of kanamycin or G418. At this time, tissue pieces which are growing well are subcultured to fresh selection medium.

F. Growth of Somatic Embryos. Somatic embryos form on these tissue pieces. Somatic embryos are explanted to fresh medium (non selective).

G. Germination. When the embryos begin to differentiate and germinate, i.e. at the point where they are beginning to form roots and have two or three leaves, they are transferred to boxes containing growth medium. Growth is allowed to proceed until the plantlet has 6 to 8 leaves, at which time it is removed from the agar medium.

H. Growth of Plantlet. The plantlet is placed in potting soil, covered with a beaker to maintain humidity and placed in an incubator for 4 to 8 weeks. At this time, the beaker is removed and the plant is transferred to the greenhouse. The plants grow in the greenhouse, flower and set seed.

Example 10: Transformation of Cotton Suspension Culture Cells to a Hygromycin-resistant Non-tumorous Phenotype.

The same procedure as in Example 9 is followed except where noted. Different transforming Agrobacteria are used and the plant selection medium contains an antibiotic appropriate for the selection of transformed plant tissue.

A. Growth of Plant Tissue. As in Example 8, part A.

B. Description of the *Agrobacterium* Vector. The transforming Agrobacteria contain the T-DNA containing binary vector pCIB715 (Rothstein et al., 1987) as well as the vir plasmid. The T-DNA of pCIB715 contains a chimeric gene composed of the promoter and terminator from the CaMV 35S transcript (Odell et al., 1985) and the coding sequence for hygromycin B phosphotransferase (Gritz and Davies, 1983).

C. Growth of Agrobacteria. Agrobacteria containing pCIB715 are grown on YEB containing kanamycin (50 µg/ml).

D. Infection. Transformation is accomplished as detailed in Example 8 with the change that the 1 ml aliquots resulting in part (c) are plated immediately on medium containing selective antibiotics. Selection medium contains 50 µg/ml hygromycin. Expression of the chimeric hygromycin gene in transformed plant tissue allows the selection of this tissue on medium containing hygromycin.

E. Growth of Transformed Tissue. As in Example 9, part E except that the antibiotic hygromycin is used in the plant selection growth medium.

Example 11: Transformation of Plant Cells by Microprojectile Bombardment

Another method to introduce foreign DNA sequences into plant cells comprises the attachment of said DNA to particles which are then forced into plant cells by means of a shooting device as described by Klein et al. (1988). Any plant tissue or plant organ may be used as the target for this procedure, including but not limited to embryos, apical and other meristems, buds, somatic and sexual tissues in vivo and in vitro. Transgenic cells and callus are selected following established procedures known in the art. Targeted tissues are induced to form somatic embryos or regenerate shoots to give transgenic plants according to established procedures known in the art. The appropriate procedure may be chosen in accordance with the plant species used.

The regenerated plant may be chimeric with respect to the incorporated foreign DNA. If the cells containing the foreign DNA develop into either micro- or macrospores, the integrated foreign DNA will be transmitted to sexual progeny. If the cells containing the foreign DNA are somatic cells of the plant, non-chimeric transgenic plants are produced by conventional methods of vegetative propagation either in vivo, i.e. from buds or stem cuttings, or in vitro following established procedures known in the art. Such procedures may be chosen in accordance with the plant species used.

Example 12: Transformation of Plant Cells by Injection.

Transfer of DNA into plant cells is also achieved by injection into isolated protoplasts, cultured cells and tissues as described by Reich et al. (1986 a and b) and injection into meristematic tissues of seedlings and plants as described by de la Peña et al. (1987), Graves and Goldman (1986), Hooykaas-Van Slogteren et al. (1984) and Grimsley et al. (1987 and 1988). Transgenic plants and progeny therefrom are obtained by conventional methods known in the art.

Example 13: Preparation of a Special Type of Callus of *Zea mays*, Elite Inbred Line Funk 2717

Zea mays plants of the inbred line Funk 2717 are grown to flowering in the greenhouse, and self pollinated. Immature ears containing embryos approximately 2 to 2.5 mm in length are removed from the plants and sterilized in 10 % Clorox® solution for 20 minutes. Embryos are aseptically removed from the kernels and placed with the embryo axis downwards on OMS medium containing 0.1 mg/liter 2,4-D, 6 % (w/v) sucrose and 25 mM L-proline solidified with 0.24 % (w/v) Gelrite® (initiation medium). After two weeks' culture in the dark at 27°C, the callus developing on the scutellum is removed from the embryo and placed on B5 medium (Gamborg et al., 1968), containing 0.5 mg/liter 2,4-D and solidified with 0.24 % (w/v) Gelrite®. The callus is subcultured every 2 weeks to fresh medium. After a total of 8 weeks after placing the embryos on the initiation medium, the special type of callus is identified by its characteristic morphology. This callus is subcultured further on the same medium. After a further period of 2 months, the callus is transferred to, and serially subcultured on, N6 medium containing 2 mg/liter 2,4-D and solidified with Gelrite®.

Example 14: Preparation of a Suspension Culture of *Zea mays* Elite Inbred Line Funk 2717

The callus described in this example is subcultured for a total of at least 6 months. The type of callus chosen for subculture is relatively non-mucilaginous, granular and very friable, such that it separates into

small individual cell aggregates upon placing into liquid medium. Cultures containing aggregates with large, expanded cells are not retained. Approximately 500 mg aliquots of the special callus of *Zea mays* elite inbred line Funk 2717 are placed into 30 ml of N6 medium containing 2 mg/liter 2,4-D in 125 ml Delong flasks. After 1 week of culture at 26°C in the dark on a gyrotory shaker (130 rpm, 2.5 cm throw) the medium is replaced with fresh medium. The suspensions are again subcultured in this way after another week. At that time, the cultures are inspected, and those which do not show large numbers of expanded cells are retained. Suspension cultures containing aggregates with large, expanded cells are discarded. The preferred tissue consists of densely cytoplasmic dividing cell aggregates which have a characteristically smoother surface than the usual type of cell aggregates. The cultures retained have at least 50 % of the cells represented in these small aggregates. This is the desired morphology. These suspensions also have a rapid growth rate, with a doubling time of less than 1 week. The suspension cultures are subcultured weekly by transferring 0.5 ml packed cell volume (PCV: settled cell volume in a pipette) into 25 ml of fresh medium. After 4 to 6 weeks of subculture in this fashion, the cultures increase 2 to 3 fold per weekly subculture. Cultures in which more than 75 % of the cells are of the desired morphology are retained for further subculture. The lines are maintained by always choosing for subculture the flask whose contents exhibit the best morphology. Periodic filtration through 630 µm pore size stainless steel sieves every 2 weeks is used in some cases to increase the dispersion of the cultures, but is not necessary.

A *Zea mays* suspension culture is deposited with the American Type Culture Collection (ATCC) accession number 40326. This deposition was made in accordance with the Budapest Treaty (date of deposit: May 20, 1987).

Example 15: Preparation of Protoplasts from Suspension Cultures of *Zea mays*

1 to 1.5 ml PCV of the suspension culture cells prepared as in the preceding example are incubated in 10 to 15 ml of a filter-sterilized mixture consisting of 4 % (w/v) cellulase RS with 1 % (w/v) Rhozyme in

KMC (8.65 g/liter KCl, 16.47 g/liter $MgCl_2.6\ H_2O$ and 12.5 g/liter $CaCl_2.2\ H_2O$, 5 g/liter MES, pH 5.6) salt solution. Digestion is carried out at 30°C on a slow rocking table, for a period of 3 to 4 hours. The preparation is monitored under an inverted microscope for protoplast release. The protoplasts which are released are collected as follows: The preparation is filtered through a 100 µm mesh sieve, followed by a 50 µm mesh sieve. The protoplasts are washed through the sieves with a volume of KMC salt solution equal to the original volume of enzyme solution. 10 ml of the protoplast preparation is placed in each of several plastic disposable centrifuge tubes, and 1.5 to 2 ml of 0.6 M sucrose solution (buffered to pH 5.6 with 0.1 % (w/v) MES and KOH) layered underneath. The tube is centrifuged at 60 to 100 x g for 10 minutes, and the protoplasts banding at the interface are collected using a pipette and placed in a fresh tube. The protoplast preparation is resuspended in 10 ml of fresh KMC salt solution, and centrifuged for 5 minutes at 60 to 100 x g. The supernatant is removed and discarded, and the protoplasts resuspended gently in the drop remaining, and then 10 ml of a 13/14 strength KMC solution gradually added. After centrifuging again for 5 minutes, the supernatant is again removed and the protoplasts resuspended in a 6/7 strength KMC solution. An aliquot is taken for counting, and the protoplasts again sedimented by centrifugation. The protoplasts are resuspended at 10^7 (ten million) per ml in KM-8p medium in 0.5 M mannitol containing 6 mM $MgCl_2$ or in any other appropriate medium for use in transformation as described in the following examples.

Example 16: Transformation of *Zea mays* Protoplasts by Electroporation

(A) All steps except the heat shock are carried out at room temperature (22° to 28°C). The protoplasts from the last step of the example immediately above are resuspended in 0.5 M mannitol containing 0.1 % (w/v) MES and 6 mM $MgCl_2$. The resistance of this suspension is measured in the chamber of a Dialog Electroporator® (DIA-LOG GmbH, D-4000 Düsseldorf 13, Federal Republic of Germany) and adjusted to 1 to 1.2 kΩ using a 300 mM $MgCl_2$ solution. The protoplasts are heat-shocked by immersing the tube containing the sample in a water bath at 45°C for 5 minutes, followed by cooling to room temperature on ice. 4 µg of linearized plasmid containing a plant-selectable hygromycin resistance gene such as described by Roth-

stein et al. (1987) or chimeric gene constructs as described in Examples 32, 36, 41 and 46, and 20 µg of calf thymus carrier DNA are added to aliquots of 0.25 ml of this suspension. 0.125 ml of a 24 % (w/v) polyethylene glycol (PEG) solution (MW of PEG 8000) in 0.5 M mannitol containing 30 mM $MgCl_2$ are added to the protoplasts. The mixture is mixed well but gently, and incubated for 10 minutes. The sample is transferred to the chamber of the electroporator and samples pulsed 3 times at 10 second intervals at initial voltages of 1500, 1800, 2300 or 2800 Vcm^{-1}, and an exponential decay time of 10 µs.

The protoplasts are cultured as follows: The samples are plated in 6 cm Petri dishes at room temperature. After a further 5 to 15 minutes, 3 ml of KM-8p medium containing 1.2 % (w/v) SeaPlaque® agarose and 1 mg/liter 2,4-D are added. The agarose and protoplasts are mixed well, and the medium allowed to gel.

B. The procedure of A is repeated with one or more of the following modifications:

(1) The resistance of the protoplast preparation is adjusted to 0.5 to 0.7 kΩ.

(2) The PEG used is PEG with a molecular weight of 4000.

(3) No PEG is added, or one half volume of 12 % (w/v) PEG is added.

(4) The pulses are applied at intervals of 3 seconds.

(5) The protoplasts are plated after the electroporation in dishes placed on a plate cooled to a temperature of 16°C.

(6) The protoplasts are placed in tubes after the electroporation step, washed with 10 ml of 6/7 strength KMC solution or with W5 solution (comprised of 380 mg/liter KCl; 18.375 g/liter $CaCl_2 \cdot 2H_2O$; 9 g/liter NaCl; 9 g/liter glucose; pH 6.0), then collected by centrifugation at 60 x g for 10 minutes, resuspended in 0.3 ml of KM medium, and plated as in A.

(7) The calf thymus carrier DNA is not added.

Example 17: Transformation of *Zea mays* Protoplasts by Treatment with Polyethylene Glycol (PEG)

A. The protoplasts are resuspended at the last step of example 15 in a 0.5 M mannitol solution containing 12 to 30 mM $MgCl_2$. A heat shock of 45°C for 5 minutes is given as described in Example 16. The protoplasts are distributed in aliquots for transformation in centrifuge tubes, 0.3 ml of suspended protoplasts per tube. During the next 10 minutes the following are added: DNA (as in Example 16A) and PEG solution (40 % (w/v) MW 6000; containing 0.1 M $Ca(NO_3)_2$ and 0.4 M mannitol; pH 8 - 9 with KOH) to give a final concentration of 20 % PEG. The aliquots are incubated for 30 minutes with occasional gentle shaking and then the protoplasts are placed in petri dishes (0.3 ml original protoplast suspension per 6 cm diameter dish) and cultured as described in Example 16A.

B. The procedure of A above is repeated, but the protoplasts are washed after 30 minutes of incubation in the PEG solution by adding 0.3 ml of W5 solution 5 times at 2 to 3 minutes intervals. The protoplast suspension is centrifuged, the supernatant removed, and the protoplasts are cultured as for Example 16 (A).

C. The procedures of A and B above are repeated with the modification that the final concentration of PEG is between 13 and 25 % (w/v).

Example 18: Regeneration of Callus from Protoplasts

The plates containing the protoplasts in agarose are placed in the dark at 26°C. After 14 days, cell colonies arise from the protoplasts. The agarose containing the colonies is transferred to the surface of a 9 cm diameter petri dish containing 30 ml of N6 medium containing 2 mg/liter 2,4-D, solidified with 0.24 % (w/v) Gelrite®. This medium is referred to as 2N6. The callus is cultured further in the dark at 26°C and callus pieces subcultured every 2 weeks onto fresh solid 2N6 medium.

Example 19: Selection of Transformed Callus of *Zea mays*

Example 18 is repeated with the modification that 100 mg/liter or 200 mg/liter hygromycin B is added to the 2N6 medium in order to select for transformed cells.

Example 20: Regeneration of Corn Plants

A. Callus is placed on 2N6 medium for maintenance and on ON6 (comprising N6 medium lacking 2,4-D) and N61 medium (comprising N6 medium containing 0.25 mg/liter 2,4-D and 10 mg/liter kinetin) to initiate regeneration. Callus growing on ON6 and N61 media is grown in the light (16 hrs/day light of 800 to 8000 lx from white fluorescent lamps). Callus growing on N61 medium is transferred to ON6 medium after 2 weeks, as prolonged time on the N61 medium is detrimental. The callus is subcultured every 2 weeks even if the callus is to be transferred again on the same medium formulation. Plantlets appear in about 4 to 8 weeks. Once the plantlets are at least 2 cm tall, they are transferred to ON6 medium in GA7 containers. Roots form in 2 to 4 weeks, and when the roots look well-formed enough to support growth, the plantlets are transferred to soil in peat pots, under a light shading for the first 4 to 7 days. It is often helpful to invert a clear plastic cup over the transplants for two to three days to assist hardening off. Once the plants are established, they are treated as normal corn plants and grown to maturity in the greenhouse. In order to obtain progeny plants are self pollinated or crossed with wild type.

B. The procedure of A above is repeated with the modification that 100 mg/liter or 200 mg/liter hygromycin B is added to the medium used to maintain the callus.

Example 21: Preparation of Embryogenic Suspensions from Tissue of *Dactylis glomerata* L. (orchard gras)

A. Embryogenic callus is initiated from basal sections of the youngest leaves of greenhouse-grown *Dactylis glomerata* L. as described by Hanning and Conger (1982). The leaves are surface sterilized by immersion in a

1:10 dilution of Clorox® solution [a solution of 5.25 % (w/v) sodium hypochlorite; The Clorox Company, Oakland, CA 94623, USA] for about 10 minutes and then cut aseptically into small segments of 1 to 5 mm in length or in diameter. These segments are placed on sterile SH-30 medium containing 0.8 % (w/v) agarose as a gelling agent. Callus and/or embryogenic structures appear within 2 to 6 weeks after plating, upon culture at about 25°C. Embryogenic callus is maintained by subculturing onto fresh SH-30 medium every 2 to 4 weeks and culturing in the dark at 25°C.

B. Embryogenic suspension cultures are initiated by placing approximately 0.5 g fresh weight of embryogenic callus into 50 ml of liquid medium described by Gray and Conger (1985) containing 45 µM dicamba and 4 g/liter casein hydrolysate. The suspension cultures are grown at 27°C under a 16 hours light (3300 lx), 8 hours dark photoperiod on a gyrotory shaker at about 130 rpm in 125 ml Delong flasks sealed with a metal cap and parafilm®. After approximately four weeks the large clumps are allowed to settle for about 30 seconds and 10 ml aliquots of the supernatant medium containing small cell clusters are removed and transferred to 50 ml of fresh medium. This proceess is repeated every 3 to 4 weeks using the most successful cultures as judged by smaller clump size and better quality based on the presence of small, cytoplasmic cells. After 5 to 8 transfers the suspensions are essentially free of non embryogenic cells and the majority of the embryogenic cell clusters are quite small (150 to 2000 µm).

Example 22: Isolation and Purification of *Dactylis glomerata* L. Protoplasts

Protoplast are prepared from embryogenic suspension cultures of the preceding example by aseptically filtering the cells on a Nalgene® 0.2 µm filter unit and then adding 0.5 g cells (fresh weight) to each 12.5 ml protoplast enzyme mixture in a petri dish. The enzyme mixture consists of 2 % (w/v) Cellulase RS, 7 mM $CaCl_2 \cdot H_2O$, 0.7 mM $NaH_2PO_4 \cdot H_2O$, 3 mM MES (pH 5.6), glucose (550 mOs/kg H_2O of pH 5.6), and is filter sterilized. The mixture is swirled on an orbital shaker at about 50 rpm in dim (< 400 lx) light for about 4 to 5 hours. The digest is then sieved through a stainless steel sieve (100 µm mesh size) and

distributed into 12 ml centrifuge tubes which are centrifuged at about 60 to 100 x g for about 5 minutes. The protoplast-containing sediment is then washed three times with protoplast culture medium KM-8p adjusted to 550 mOs/kg H_2O with glucose. At this point a flotation step may be included for further purification of the protoplasts. In this case, the washed protoplasts are layered atop 10 ml of KM-8p culture medium adjusted to 700 mOs/kg H_2O with sucrose. After centrifugation at 60 to 100 x g for about 10 minutes, protoplasts banding at the interface are collected using a fine pipette. Finally, the protoplasts are resuspended in 1 to 2 ml KM-8p culture medium and sieved through a stainless mesh screen (20 µm mesh size). The protoplasts released are collected and washed and resuspended in KM-8p medium for culture or in osmotically adjusted medium suitable for transformation.

Example 23: *Dactylis glomerata* L. Protoplast Culture and Growth of Callus

A. The purified protoplasts are plated at a density of about 5 x 10^5 protoplasts/ml in KM-8p culture medium containing 1.3 % (w/v) SeaPlaque® agarose (FMC Corp., Marine Colloids Division, Rockland, Maine, USA) and 30 to 40 % (v/v) of conditioned medium (obtained from 3 to 4 week-old *D. glomerata* L. embryogenic suspension cultures by filtering the medium through a sterile Nalgene® 0.2 µm filter, making the medium 550 mOs/kg H_2O by addition of glucose, and again filter sterilizing). The plates are then placed in the dark at a constant temperature of 28°C. After 10 to 14 days the agarose is cut into wedges and placed into 'bead culture' as described by Shillito et al. (1983) using 20 ml SH-45 suspension culture medium with 3 % (w/v) sucrose per 3 ml original agarose embedded culture. The plates are put on a platform shaker and agitated at about 50 rpm in light at 670 lx. New suspension cultures are formed as the colonies grow out of the agarose and release cells into the liquid medium. The resultant suspension cultured cells are plated onto agar-solidified SH-30 medium and placed in the dark at 25°C until callus is formed.

B. Protoplasts are cultured as described in A above except that the culture medium contains no conditioned medium.

Example 24: Transformation of *Dactylis glomerata* L. Protoplasts by Means of Electroporation

A. Immediately after purification of the protoplasts, electroporation is performed according to Shillito et al. (1985) using linearized plasmid as described in Example 16. The protoplasts are resuspended after the last wash at a density of about 7×10^6 protoplasts/ml in the electroporation buffer (0.4 M mannitol, 6 mM $MgCl_2$). The protoplasts are placed in 0.7 ml aliquots in 10 ml plastic centrifuge tubes. Plasmid DNA to give a final concentration of 10 µg/ml and sonicated calf thymus DNA (Sigma) to give 50 µg/ml are added to the tubes. Then 0.38 ml PEG solution [24 % (w/v) PEG 6000 in 0.4 M mannitol, 30 mM $MgCl_2$, 0.1 % (w/v) MES (pH 5.6)] is added and the solution gently mixed. The protoplast suspension is transferred into the chamber of a Dialog® Electroporator and 10 pulses of 3250 V/cm initial voltage and exponential decay constant of 10 µs applied at 30 s intervals. The sample is removed from the chamber, and placed in a 10 cm diameter petri dish. 10 ml of KM-8p medium containing 1.2 % (w/v) SeaPlaque® agarose is added, the protoplasts distributed evently throughout the medium, and the agarose allowed to gel.

B. Example 24A is repeated except that the initial voltage used is 2500 Vcm^{-1}, 3000 Vcm^{-1}, 3500 Vcm^{-1}, 4000 Vcm^{-1} or 5000 Vcm^{-1}.

C. Examples 24 A and B are repeated except that PEG of MW 4000 or PEG of MW 8000 is used.

D. Examples 24 A to C are repeated except that the final PEG concentration is between 10 % and 30 % (w/v).

Example 25: Transformation of *Dactylis glomerata* L. Protoplasts by Treatment with Polyethylene Glycol (PEG)

A. PEG mediated direct gene transfer is performed according to Negrutiu et al. (1987). The protoplasts are suspended following the last wash in 0.5 M mannitol containing 15 mM $MgCl_2$ at a density of about 2×10^6 per ml. The protoplast suspension is distributed as 1 ml aliquots into 10 ml plastic centrifuge tubes. The DNA is added as

described in Example 24, and then 0.5 ml of the PEG solution added [40 % (w/v) PEG 4000 in 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 7.0]. The solutions are mixed gently and incubated for 30 minutes at room temperature (about 24°C) for 30 minutes with occasional shaking. 1.4 ml of the wash solution is then added, and the contents of the tube gently mixed. The wash solution consists of 87 mM mannitol, 115 mM $CaCl_2$, 27 mM $MgCl_2$, 39 mM KCl, 7 mM Tris/HCl and 1.7 g/liter myo-inositol, pH 9.0. Four further 1.4 ml aliquots of wash solution are added at 4 minute intervals, with mixing after each addition. The tube is then centrifuged at about 60 x g for about 10 minutes, and the supernatant discarded. The sedimented protoplasts are taken up in 1 ml KM-8p culture medium, and placed in a 10 cm Petri dish. 10 ml of KM-8p medium containing 1.2 % (w/v) SeaPlaque® agarose is added. The protoplasts are evenly distributed throughout the medium, and the agarose allowed to gel.

B. The procedure of A above is repeated with one or more of the following modifications:

(1) The pH of the wash solution is adjusted to 5.6 or 7.0.

(2) The PEG used is PEG of MW 2000, 6000 or 8000.

(3) The wash medium consists of (a) 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 6.0 with KOH, of (b) 0.2 M $CaCl_2$, 0.1 % (w/v) MES, pH 6.0 with KOH, or of (c) 0.2 M $CaCl_2$, 7 mM Tris/HCl, pH 9.0 with KOH.

Example 26: Transformation of *Dactylis glomerata* L. Protoplasts after Heat Shock Treatment

Transformation is carried out as described in Examples 24 or 25, except that the protoplasts are treated at 45°C for about 5 minutes prior to distribution of the aliquots into tubes for transformation or after distribution of the aliquots, and before addition of the PEG.

Example 27: Selection of Transformed Colonies

A. The culture plates (Petri dishes) containing the protoplasts from examples 24 to 26 are incubated for 10 days in the dark at about 25°C and then cut into 5 equal slices for 'bead cultures' (Shillito et al., 1983). 4 of the slices are placed each into 20 ml SH-45 culture medium with

4 g/liter casein hydrolysate and 20 µg/ml hygromycin B. The fifth slice is put into 20 ml of the same medium but without hygromycin B as a non-selected control. After 4 to 5 weeks the putative transformed protoplast-derived cell colonies growing in hygromycin B are cut out of the agarose and placed into a 19 mm Petri dish with 2 ml of liquid SH-45 medium containing 20 µg/ml hygromycin B, which is agitated at about 50 rpm on an orbital shaker. After another 4 to 5 weeks all colonies which grow to make new suspensions are transferred into 125 ml Erlenmeyer flasks and grown in a manner similar to the parent suspension culture, except that 20 µg/ml hygromycin B is included in the medium.

The new suspensions are subcultured every 1 to 3 weeks using SH-45 medium containing 4 g/liter casein hydrolysate and 20 µg/ml hygromycin B. Cells from these suspensions are also plated on solidified SH-30 medium containing 20 µg/ml hygromycin B and incubated at about 25°C in the dark. Calli grown from the plated cells are subcultured every two weeks onto fresh medium. The cells which grow in the presence of hygromycin B are presumed to be transformants.

B. Selection is carried out as described in Example 27 (A) except that the protoplast-derived cell colonies growing in hygromycin B - containing medium are placed on agar plates of SH-30 medium containing 20 µg/ml hygromycin B and incubated at about 25°C in the dark.

Example 28: Regeneration of Transformed *Dactylis glomerata* L. Plants

A. *D. glomerata* L. callus (obtained as described in Example 27) derived from protoplasts is grown on solidified SH-30 medium, and subcultured every two weeks. Any embryos which form are removed and plated on germination medium (SH-0) and placed in the light (3700 to 4600 lx). Germination of these embryos occurs in 1 to 4 weeks and the resultant plantlets are placed on SH-0 medium in the light to form root systems. They are moved into the greenhouse at the six to twelve leaf stage, and hardened off gradually.

B. Callus (obtained as described in Example 27) derived from protoplasts is grown on SH-0 medium solidified with 0.24 % (w/v) GelRite® in the light (3700 to 4600 lx), and subcultured every two weeks. The resultant plantlets are placed on a 1:1 mixture of SH-0 and OMS media solidified with a combination of 0.12 % (w/v) GelRite® and 0.4 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.

C. Small plantlets are obtained as described in A and B above, and are placed on OMS medium solidified with 0.8 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.

D. Small plantlets are obtained as described in A above and are placed on a 1:1 mixture of SH-0 and OMS media solidified with a combination of 0.12 % (w/v) GelRite® and 0.4 % (w/v) agar in the light to form root systems. They are moved to the greenhouse at the six to twelve leaf stage, and hardened off gradually.

Example 29: Transformation and Regeneration of Tomato

Tomatoes are transformed (Nelson et al., 1988) with engineered *A. tumefaciens* bearing a plant selectable marker (eg. kanamycin-resistance) and the chimeric gene encoding the protease inhibitor of interest. Leaf disk transformation of tomato with *A. tumefaciens* is according to McCormick et al. (1986).

Example 30: Transformation and Regeneration of Potato

Potatoes are transformed (Stockhaus et al., 1987) with engineered *A. tumefaciens* bearing a plant selectable marker (eg. kanamycin-resistance) and the chimeric gene encoding the protease inhibitor of interest.

IV. Results

Example 31: Bioassay of Transformed Cotton

Heliothis virescens eggs laid on sheets of cheesecloth are obtained from the Tobacco Insect Control Laboratory at North Carolina State University, Raleigh, North Carolina. The cheesecloth sheets are transferred to a large covered glass beaker and incubated at 29°C with wet paper towels to maintain humidity. The eggs hatch within three days. As soon as possible after hatching, the larvae (one larva per cup) are transferred to covered small plastic cups. Each cup contains cotton leaf discs. Larvae are transferred using a fine bristle paint brush.

Leaf discs one cm in diameter are punched from leaves of cotton plants and placed on a circle of wet filter paper in the cup with the larva. At least 6 to 10 leaf discs, representing both young and old leaves, are tested from each plant. Leaf discs are replaced at two day intervals, or as necessary to feed the larvae. Growth rates (size or combined weight of all replica worms) and mortality of larvae feeding on leaves of transformed plants are compared with those of larvae feeding on untransformed cotton leaves.

Larvae feeding on discs of cotton transformed with a gene encoding a protease inhibitor show a large decrease in growth rate and 100 % mortality compared with controls.

Example 32: Maize Resistant to Damage by *Diabrotica* (Corn Root Worm) through Expression of Egg White Cystatin

A. Construction of the pCIB715/710-Cystatin Vector. The cystatin gene is synthesized as described in Example 3 C. The 363 bp DNA sequence is purified and ligated into the BamHI site of the pCIB710 vector as described in Example 5. The resulting vector is denoted as pCIB710-cystatin. This vector is cut with EcoRI and the resulting DNA is treated with alkaline phophatase. Plasmid pCIB715 (Rothstein et al., 1987) is cut with EcoRI and ligated with the pCIB710-cystatin from above. The resulting vector is named pCIB715/710-cystatin.

B. Transformation and Regeneration of Maize. Maize tissue is transformed with the pCIB710 vector or, preferably, the pCIB715/710 vector carrying the cystatin gene insert and plants are regenerated as described in Examples 16 to 20. For controls, plants transformed with the pCIB710 vector or the pCIB715 vector without the gene insert are prepared in the same way. Transformed plant tissue is selected on the appropriate antibiotic. The initial plants are self-fertilized and seed (T1 seed) is obtained.

C. Testing Plants for Cystatin Expression. Plants grown from the T1 seed are analyzed for the presence and expression of the cystatin gene using several tests.

(1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the cystatin gene labelled with ^{32}P by nick translation [see Maniatis et al. (1982) for techniques]. The presence of the cystatin gene is detected by a band of approximately 363 bp which hybridizes to the probe.

(2) RNA is detected by the Northern blot procedure (Maniatis et al., 1982) as a band of approximately 380 bases which hybridizes with the ^{32}P-cystatin gene described above.

(3) Cystatin protein is detected using standard immunological techniques with a polyclonal rabbit antibody raised against commercially (Sigma) available cystatin.

(4) Cystatin activity is detected by immunopurifying material from the plant extracts using the rabbit polyclonal anti-cystatin antibody and protein A Sepharose and assaying the isolated material for the ability to inhibit proteolysis of ^{14}C-BSA by papain and by corn root worm gut homogenates as described in Example 1.

D. Resistance of Transformed Corn Plants to *Diabrotica* Damage. T1 seedlings are planted in coarse vermiculite in 100 mm Petri dishes (5 per dish, 5 dishes). When the second leaves on the seedlings emerge, each dish is infested with 20 second instar *Diabrotica* larvae. After seven days, the number and weight of the survivors is measured along with the weight of the washed roots of the corn plant. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root weight relative to insect-free plants when plants expressing the cystatin gene are compared to control plants transformed with the vector without gene insert or untransformed plants.

Example 33: Potato and Tomato Resistant to Damage by *Leptinotarsa decemlineata* through Expression of Cystatin

A. Construction of the pCIB10-Cystatin Vector. The cystatin gene is synthesized and ligated into vector pCIB710 as described in Example 32 A. The cystatin gene plus the 35S CaMV promoter are subcloned out of the pCIB710 vector into the pCIB10 vector by digesting with XbaI and EcoRI, isolating the 1593 bp fragment and ligating this fragment with XbaI, EcoRI digested pCIB10 to make the pCIB10-cystatin vector.

B. Transformation and Regeneration of Plants. The pCIB10-cystatin vector or, preferably, the pCIB715/710-cystatin vector is introduced into *A. tumefaciens* carrying a virulence plasmid such as LBA 4404 (Example 5) or pCIB542. pCIB542 is an engineered *A. tumefaciens* vir plasmid derived from pTiBo542 (Hood et al., 1986). pCIB542 has the bacterial kanamycin resistence gene replaced by a bacterial streptomycin/spectinomycin resistence gene. The strain carrying both pCIB10-cystatin or pCIB715/710 cystatin and pCIB542 is used to produce transformed tomato plants according to the method of Fischhoff et al. (1987), or as in Example 29.

Potato plants containing pCIB10-cystatin or pCIB715/710-cystatin are obtained by the method of Stockhaus et al. (1987), Example 30.

C. Testing Plants of Cystatin Expression. Testing of transformants for cystatin expression is carried out as described above for maize.

D. Resistance of Transformed Plants to Damage by *L. decemlineata*. Ten four week old plants are each infested with five second instar *L. decemlineata* larvae. Larvae are allowed to feed for four days at which time insect mortality, insect weight gain and amount of damage to the plant are scored. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0,05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in plant damage when plants expressing the cystatin gene are compared to control plants transformed with the vector without the gene insert or untransformed plants.

Example 34: *Dactylis glomerata* (Orchard Grass) Resistant to Damage by Coleopterans through Expression of Cystatin

A. Construction of Vector. pCIB710-cystatin and pCIB715/710 cystatin are prepared as described in Example 32A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Examples 24 to 28 using pCIB710-cystatin and pCIB715/710-cystatin.

C. Testing Plants for Cystatin Expression. Testing of transformants for cystatin expression is carried out as described in Example 32C.

D. Resistance of Transformed Plants to Damge by *Diabrotica undecimpunctata*. T1 seedlings are planted in fine soil in 100 mm Petri dishes (10/dish, 5 dishes). When the second leaves on the seedlings emerge, each of five dishes is infested with 20 second instar *D. undecimpunctata* larvae. After seven days, the number and weight of the survivors is measured along with the weight of the washed roots of the plant. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root mass relative to insect-free plants when plants expressing the cystatin gene are compared to control plants transformed with the vector without the gene insert or untransformed plants.

Example 35: Cotton Resistant to Damage by *Anthonomus grandis* (Boll Weevil) through Expression of Cystatin

A. Construction of Vector. pCIB715/710 cystatin and pCIB10-cystatin are prepared as described in Examples 32A and 33A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained using pCIB715/710-cystatin or pCIB10-cystatin in *Agrobacterium* as described in Examples 7 to 10.

C. Testing Plants for Cystatin Expression. Testing of transformants for cystatin expression is carried out as described in Example 32C.

D. Resistance of Transformed Plants to Damage by *A. grandis*. Ten transformed plants are grown until bolls start to form. Each plant is infested with three adult female *A. grandis*. Damage to plants is rated after one week and surviving adults are removed. Damage by larva, larval numbers and weight per plant are measured at weekly intervals for four weeks. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in damage ratings, decrease in larval number, or decrease in larval weight when plants expressing the cystatin gene are compared to control plants transformed with the vector without the gene insert or untransformed plants.

Example 36: Maize Resistant to Damage by Lepidopterous Larvae through Expression of Soybean Kunitz Trypsin Inhibitor

A. Construction of the pCIB715/710-KTI Vector. The soybean trypsin inhibitor cDNA is obtained by the method of Hoffman et al. (1984). The 652 bp fragment is modified so that the sequence begins at the methionine start codon (44 in the original fragment) and codons for the missing three amino acids plus a stop codon are added to the 3'end. BamHI linkers are added to both ends. This construction is accomplished by digesting the 652 bp fragment with Eco57I and BstXI and isolating the resulting 517 bp fragment following agarose gel electrophoresis. The oligonucleotides shown in Figure 11 are synthesized. Phosphates are added to the 5' ends

of the 517 bp fragment and oligonucleotides 3M and 5C and all fragments are ligated as described above. The resulting 630 bp fragment is isolated and ligated into the BamHI site of the pCIB710 vector as described above. The resulting vector is denoted pCIB710-KTI.

B. Transformation and Regeneration of Maize. Maize tissue is transformed with the pCIB710 vector or, preferably, the pCIB715/710 vector carrying the soybean Kunitz trypsin inhibitor gene insert and plants are regenerated as described in Examples 16-20. For controls, plants transformed with the pCIB710 vector or the pCIB715 vector without the gene insert are prepared in the same way. Transformed plant tissue is selected on the appropriate antibiotic. The initial plants are self-fertilized and seed (T1 seed) is obtained.

C. Testing Plants for Soybean Kunitz Trypsin Inhibitor Expression. Plants grown from the T1 seeds are analyzed for the presence and expression of the soybean Kunitz trypsin inhibitor gene using the following tests.

(1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the soybean Kunitz trypsin inhibitor gene labelled with ^{32}P by nick translation [see Maniatis et al. (1982) for techniques]. The presence of the soybean Kunitz trypsin inhibitor gene is detected by a band of approximately 630 bp which hybridizes to the probe.

(2) RNA is detected by the Northern blot procedure as a band of about 650 bases which hybridizes with the ^{32}P-soybean Kunitz trypsin inhibitor gene described above.

(3) Soybean Kunitz trypsin inhibitor protein is detected using standard immunological techniques with a polyclonal rabbit antibody raised against commercially (Sigma) available soybean Kunitz trypsin inhibitor which has been repurified by reverse phase HPLC on a Vydac phenyl column.

(4) Soybean Kunitz trypsin inhibitor activity is detected by immunopurifying material from the plant extracts using the rabbit polyclonal anti-soybean Kunitz trypsin inhibitor antibody and protein A Sepharose and assaying the isolated material for the ability to inhibit proteolysis of ^{14}C-BSA by trypsin using the assay described in Example 1.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seeds are germinated and leaf pieces obtained from seedlings at the four leaf stage are used to feed neonatal *Ostrinia nubilalis* or *Heliothis zea* larvae. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from expressing the soybean Kunitz trypsin inhibitor gene are compared to those of control plants transformed with the vector without the gene insert or those of untransformed plants.

Example 37: Cotton Resistant to Damage by Lepidopterous Larvae through Expression of Soybean Kunitz Trypsin Inhibitor

A. Construction of the pCIB10-KTI Vector. The soybean Kunitz trypsin inhibitor gene together with the 35S CaMV promoter is removed from pCIB710-KTI (Example 36A) using appropriate restriction enzymes and ligated into pCIB10. This vector is denoted as pCIB10-KTI.

B. Transformation and Regeneration of Plants. Transformed plants are obtained using pCIB10-KTI or pCIB715/710KTI (Example 36A) in *Agrobacterium* as described in Examples 7 to 10.

C. Testing Plants for Trypsin Inhibitor Expression. Testing of transformants for soybean Kunitz trypsin inhibitor expression is carried out as described in Example 36C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis virescens*, *H. zea* or *Pectinophora gossypiella*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the soybean Kunitz trypsin inhibitor gene are compared to those of control plants transformed with the vector without gene insert or to those of untransformed plants.

Example 38: Tomato Resistant to Damage by Lepidopterous Larvae through Expression of Soybean Kunitz Trypsin Inhibitor

A. Construction of Vector. pCIB715/710-KTI and pCIB10-KTI are prepared as described in Example 36A and 37A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 33B using the pCIB10-KTI vector or the pCIB715/710-KTI vector.

C. Testing Plants for Trypsin Inhibitor Expression. Testing of transformants for soybean Kunitz trypsin inhibitor expression is carried out as described in Example 36C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis zea* or *Manduca sexta*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the

soybean Kunitz trypsin inhibitor gene are compared to those of control plants transformed with the vector without the gene insert or to those of untransformed plants.

Example 39: Tobacco Resistant to Damage by Lepidopterous Larvae through Expression of Soybean Kunitz Trypsin Inhibitor

A. Construction of Vector. The pCIB10-KTI vector is prepared as described in Example 37A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 6 using the pCIB10-KTI vector or the pCIB715/710-KTI vector (Example 36A) in *Agrobacterium*.

C. Testing Plants for Trypsin Inhibitor Expression. Testing of transformants for soybean Kunitz trypsin inhibitor expression is carried out as described in Example 36C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis zea* or *Manduca sexta*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the soybean Kunitz trypsin inhibitor gene are compared to those of control plants transformed with the vector without the gene insert or to those of untransformed plants.

Example 40: Orchard Grass Resistant to Damage by Lepidopterous Larvae Through Expression of Soybean Kunitz Trypsin Inhibitor

A. Construction of Vector. pCIB710-KTI and pCIB715/710-KTI are prepared as described in Example 36A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Examples 24 to 28 using pCIB710-KTI and pCIB715/710-KTI.

C. Testing Plants for Trypsin Inhibitor Expression. Testing of transformants for soybean Kunitz trypsin inhibitor expression is carried out as described in Example 36C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seedlings are planted in fine soil in 100 mm Petri dishes (10 per dish, 5 dishes). When the second leaves on the seedlings emerge, each dish is infested with 20 second instar larvae of *Crambus caliginosellus*. After seven days, the number and weight of the survivors is measured along with the weight of the washed grass roots. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root weight relative to insect-free plants when plants expressing the soybean Kunitz trypsin inhibitor gene are compared to control plants transformed with the vector without gene insert or untransformed plants.

Example 41: Maize Resistant to Damage by Lepidopterous Larvae through Expression of α_1-Antitrypsin

(A) Construction of the pCIB715/710-AATI Vector. The DNA sequences encoding the α_1-antitrypsin are obtained by the method of Rosenberg et al. (1984). For insertion into the BamHI site in the pCIB710 vector, the DNA fragment is digested with appropriate enzymes and the synthetic oligonucleotides necessary to create compatible ends are ligated together with BamHI digested pCIB710 using the methodology described above. The resultant vector is denoted pCIB710-AATI. This vector is cut with EcoRI and the resulting DNA is treated with alkaline phophatase. Plasmid pCIB715 (Rothstein et al., 1987) is cut with EcoRI and ligated with the pCIB710-AATI from above. The resulting vector is named pCIB715/710-AATI.

B. Transformation and Regeneration of Maize. Maize tissue is transformed with the pCIB710 vector or, preferably, the pCIB715/710 vector carrying the α_1-antitrypsin gene insert and plants are regenerated as described in Examples 16-20. For controls, plants transformed with the pCIB710 vector or the pCIB715 vector without the gene insert are prepared in the same way. Transformed plant tissue is selected on the appropriate antibiotic. The initial plants are self-fertilized and seed (T1 seed) is obtained.

C. Testing Plants for α_1-Antitrypsin Expression. Plants grown from the T1 seed are analyzed for the presence and expression of the α_1-antitrypsin gene using the following tests.

(1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the α_1-antitrypsin gene labelled with ^{32}P by nick translation. The presence of the α_1-antitrypsin gene is detected by a band of approximately 1200 bp which hybridizes to the probe.

(2) RNA is detected by the Northern blot procedure as a band of approximately 1200 bases which hybridizes with the ^{32}P-α_1-antitrypsin gene described above.

(3) α_1-antitrypsin protein is detected using standard immunological techniques with a polyclonal rabbit antibody raised against commercially (Sigma) available α_1-antitrypsin.

(4) α_1-antitrypsin activity is detected by assaying material immunopurified from the plant extracts using the rabbit polyclonal anti-α_1-antitrypsin antibody and protein A Sepharose and assaying for the ability of the isolated material to inhibit proteolysis of ^{14}C-BSA by trypsin using the assay described above.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seeds are germinated and leaf pieces obtained from seedlings at the four leaf stage are used to feed neonatal *Ostrinia nubilalis* or *Heliothis zea* larvae. Neonatal larvae are placed in individual diet cups with a

1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the α_1-antitrypsin gene are compared to those of control plants transformed with the vector without gene insert or to those of untransformed plants.

Example 42: Cotton Resistant to Damage by Lepidopterous Larvae through Expression of α_1-Antitrypsin

A. Construction of the pCIB10-AATI Vector. The α_1-antitrypsin gene plus the 35S CaMV promoter is removed from pCIB710-AATI (Example 41A) and ligated into pCIB10 using appropriate enzymes. This vector is denoted as pCIB10-AATI.

B. Transformation and Regeneration of Plants. Transformed plants are obtained using pCIB10-AATI or pCIB715/710-AATI (Example 41A) in *Agrobacterium* as described in Examples 7 to 10.

C. Testing Plants for α_1-Antitrypsin Expression. Testing of transformants for α_1-antitrypsin expression is carried out as described in Example 41C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis virescens*, *H. zea* or *Pectinophora gossypiella*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the α_1-antitrypsin gene are compared to those of control plants transformed with the vector without the gene insert or to those of untransformed plants.

Example 43: Tomato Resistant to Damage by Lepidopterous Larvae through Expression of α_1-Antitrypsin

A. Construction of Vector. pCIB715/710-AATI and pCIB10-AATI are prepared as described in Examples 41A and 42A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 33B using the pCIB10-AATI vector and the pCIB715/710-AATI vector.

C. Testing Plants for α_1-Antitrypsin Expression. Testing of transformants for α_1-antitrypsin expression is carried out as described in Example 41C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis zea* or *Manduca sexta*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the α_1-antitrypsin gene are compared to those of control plants transformed with the vector without the gene insert or to those of untransformed plants.

Example 44: Tobacco Resistant to Damage by Lepidopterous Larvae through Expression of α_1-Antitrypsin

A. Construction of Vector. pCIB715/710-AATI and pCIB10-AATI are prepared as described in Examples 41A and 42A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 6 using the pCIB10-AATI vector or the pCIB715/710-AATI vector (Example 41A) in *Agrobacterium*.

C. Testing Plants for α_1-Antitrypsin Expression. Testing of transformants for α_1-antitrypsin expression is carried out as described in Example 41C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis virescens* or *Manduca sexta*. Neonatal larvae are placed in individual diet cups with a 1 cm^2 piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the α_1-antitrypsin gene are compared to those of control plants transformed with the vector without the gene insert or to those of untransformed plants.

Example 45: Orchard Grass Resistant to Damage by Lepidopterous Larvae Through Expression of α_1-Antitrypsin

A. Construction of Vector. pCIB710-AATI and pCIB715/710-AATI are prepared as described in Example 41A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Examples 24 to 28 using pCIB710-AATI and pCIB715/710-AATI.

C. Testing Plants for α_1-Antitrypsin Expression. Testing of transformants for α_1-antitrypsin expression is carried out as described in Example 41C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seedlings are planted in fine soil in 100 mm Petri dishes (10/dish, 5 dishes). When the second leaves on the seedlings emerge, each dish is infested with 20 second instar larvae of *Crambus caliginosellus*. After seven days, the number and weight of the survivors is measured along with the weight of the washed grass roots. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root mass relative to insect-free plants when plants

expressing the α_1-antitrypsin gene are compared to control plants transformed with the vector without the gene insert or untransformed plants.

Example 46: Maize Resistant to Damage by Lepidopterous Larvae through Expression of Eglin

A. Construction of the pCIB715/710-eglin Vector. The DNA sequences encoding eglin C and eglin C mutants are obtained as described in Example 3 D. For insertion into the BamHI site of the pCIB710 vector, the DNA fragment is digested with appropriate enzymes and the synthetic oligonucleotides necessary to create compatible ends are ligated together with BamHI digested pCIB710 using the methodology described above. The resultant vector derived from the eglin C (Arg45) mutant is denoted pCIB710-eglin. This vector is cut with EcoRI and the resulting DNA is treated with alkaline phophatase. Plasmid pCIB715 (Rothstein et al., 1987) is cut with EcoRI and ligated with the pCIB710-eglin from above. The resulting vector is named pCIB715/710-eglin.

B. Transformation and Regeneration of Maize. Maize tissue is transformed with the pCIB710 vector or, preferably, the pCIB715/710 vector carrying the eglin C (Arg45) gene insert and plants are regenerated as described in Examples 16-20. For controls, plants transformed with the pCIB710 vector or the pCIB715 vector without the gene insert are prepared in the same way. Transformed plant tissue is selected on the appropriate antibiotic. The initial plants are self-fertilized and seed (T1 seed) is obtained.

C. Testing Plants for Eglin Expression. Plants grown from the T1 seed are analyzed for the presence and expression of the eglin C (Arg45) gene using the following tests.

(1) DNA is isolated and digested with BamHI; the digest is electrophoresed on a 1.5 % agarose gel. The DNA fragments are transferred to nitrocellulose and hybridized with the eglin C (Arg45) gene labeled with ^{32}P by nick translation. The presence of the eglin gene is detected by a band of approximately 230 bp which hybridizes to the probe.

(2) RNA is detected by the Northern blot procedure as a band of approximately 230 bases which hybridizes with the ^{32}P-eglin C (Arg45) gene described above.

(3) Eglin C (Arg45) mutant protein is detected using standard immunological techniques with a polyclonal rabbit antibody raised against eglin C (Arg45).

(4) Eglin activity is detected by assaying material immunopurified from the plant extracts using the rabbit polyclonal anti-eglin C (Arg45) antibody and protein A Sepharose and assaying for the ability of the isolated material to inhibit proteolysis of ^{14}C-BSA by trypsin using the assay described above.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seeds are germinated, and leaf pieces obtained from seedlings at the four leaf stage are used to feed neonatal European corn borer *(Ostrinia nubilalis)* or corn earworm *(Heliothis zea)* larvae. Neonatal larvae are placed in individual diet cups with a 1 x 1 cm piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p>0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the eglin C (Arg45) gene are compared to those of control plants transformed with the vector without gene insert or those of untransformed plants.

Example 47: Cotton Resistant to Damage by Lepidopterous Larvae through Expression of Eglin

A. Construction of the pCIB10-eglin Vector. The eglin C (Arg45) gene plus the 35S CaMV promoter are removed from pCIB710-eglin (Example 46A) and ligated into pCIB10 using appropriate enzymes. This vector is denoted pCIB10-eglin.

B. Transformation and Regeneration of Plants. Transformed plants are obtained using pCIB10-eglin or pCIB715/710-eglin (Example 46A) in *Agrobacterium* as described in Examples 7 to 10.

C. Testing Plants for Eglin Expression. Testing of transformants for eglin expression is carried out as described in Example 46C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis virescens* (tobacco budworm), *Heliothis zea* (cotton bollworm) or *Pectinophora gossypiella* (pink bollworm). Neonatal larvae are placed in individual diet cups with a 1 x 1 cm piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p>0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the eglin C (Arg45) gene are compared to those of control plants transformed with the vector without the gene insert or those of untransformed plants.

Example 48: Tomato Resistant to Damage by Lepidopterous Larvae through Expression of Eglin

A. Construction of Vector. pCIB715/710-eglin and pCIB10-eglin are prepared as described in Examples 46A and 47A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 33B using the pCIB10-eglin vector and the pCIB715/710-eglin vector.

C. Testing Plants for Eglin Expression. Testing of transformants for eglin expression is carried out as described in Example 46C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis zea* (cotton bollworm) or *Manduca sexta* (tomato/tobacco hornworm). Neonatal larvae are placed in individual diet cups with a 1 x 1 cm

piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the eglin C (Arg 45) gene are compared to those of control plants transformed with the vector without the gene insert or those of untransformed plants.

Example 49: Tobacco Resistant to Damage by Lepidopterous Larvae through Expression of Eglin

A. Construction of Vector. pCIB715/710-eglin and pCIB10-eglin are prepared as described in Examples 46A and 47A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Example 6 using the pCIB10-eglin vector or the pCIB715/710-eglin vector (Example 46A) in *Agrobacterium*.

C. Testing Plants for Eglin Expression. Testing of transformants for eglin expression is carried out as described in Example 46C.

D. Resistance of Transformed Plants to Damage from Lepidopterous Insects. Leaf disks from four week old transformed plants are fed to neonatal *Heliothis virescens* (tobacco budworm) or *Manduca sexta* (tomato/tobacco hornworm). Neonatal larvae are placed in individual diet cups with a 1 x 1 cm piece of leaf. Fifty insects are tested per group. After 5 days insect weight, insect survival, and amount of leaf eaten are rated. Resistance of transformed plants is detected by a statistically significant (Student's t test $p<0.05$) decrease in larval survival, larval weight or amount of leaf consumed when leaves from plants expressing the eglin C (Arg45) gene are compared to those of control plants transformed with the vector without the gene insert or those of untransformed plants.

Example 50: Orchard Grass Resistant to Damage by Lepidopterous Larvae through Expression of Eglin

A. Construction of Vector. pCIB710-eglin and pCIB715/710-eglin are prepared as described in Example 46A.

B. Transformation and Regeneration of Plants. Transformed plants are obtained as described in Examples 24 to 28 using pCIB710-eglin and pCIB715/710-eglin.

C. Testing Plants for Eglin Expression. Testing of transformants for eglin expression is carried out as described in Example 46C.

D. Resistance of Transformed Plants to Damage by Lepidopterous Larvae. T1 seedlings are planted in fine soil in 100 mm Petri dishes (10 per dish, 5 dishes). When the second leaves on the seedling emerge, each dish is infested with 20 second instar larvae of corn root webworm *(Crambus caliginosellus)*. After seven days, the number and weight of the survivors is measured along with the weight of the washed grass roots. Resistance of transformed plants is detected by a statistically significant (Student's t test $p>0.05$) decrease in larval weight gain, decrease in larval survival rate, or decrease in loss of root mass relative to insect-free plants when plants expressing the eglin C (Arg45) gene are compared to control plants transformed with the vector without the gene insert or untransformed plants.

Depositions:

(1) *E. coli* HB101/pML147 (DSM 4380) was deposited on January 28, 1988 with the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) in Braunschweig FRG.

The following microorganisms were deposited with the American Type Culture Collection (ATCC) in Rockville, Maryland:

(2) Plasmid pLW111 (ATCC-40235) deposited on May 14, 1986.

(3) *Zea mays* suspension culture (ATCC-40326) deposited on May 20, 1987.

(4) Plasmid p368 (ATCC-67700) deposited on May 19, 1988.

Table A

PLANT CLASSIFICATION ACCORDING TO USE

CEREALS

Monocot

Avena nuda (chinensis)	Chinese naked oat
A. sativa	Common oats
Eleusine coracan	African millet
Eragrostis tef	Tef grass
Hordeum distichum	Two-row barley
H. vulgare	Barley
Oryza sativa	Rice
Panicum italicium	Italian millet
P. miliaceum	Broomcorn millet
Pennisetum glaucum	Spiked millet
P. spicatum (americanum)	Pearl millet
Secale cereale	Rye
Sorghum vulgare	Grain sorghums
X Triticosecale	Triticale
Triticum aestivum	Common wheat
T. dicoccum	Emmer
T. durum	Abyssinian hard wheat
T. monococcum	Einkorn wheat
Zea mays	Starch, sweet corn

Dicot

Amaranthus paniculatus	Grain amaranth
Fagopyrum esculentum	Buckwheat
F. tataricum	Rye buckwheat

PROTEIN CROPS

Dicot

Arachis hypogea	Groundnut, peanut
Cajanus indicus	Pigeon pea
Cicer arietinum	Chickpea
Dolichos lablab	Hyacinth bean
Glycine gracilis	Manchurian Soya
G. max	Soyabean
G. ussuriensis	Wild soya
Lathyrus sativus	Grass pea
Lens culinaris	Lentil
Mucuna pruriens	Cowitch, Florida velvet bean
Phaseolus acutifolius	Tepary bean
P. aureus	Mung, green gram
P. lunatus	Lima bean, Sieva
P. coccineus (multiflorus)	Scarlet runner bean
P. mungo	Black gram
P. vulgaris	French, common, kidney or dwarf bean

Vicia faba	Horse bean, broad bean
Vigna angularis	Adzuki bean
V. sesquipedalis	Asparagus (yard-long bean)
V. sinensis	Cowpea

FRUIT CROPS

Dicots

Amygdalus communis	Almond
Ananas comosus	Pineapple
Artocarpus communis	Breadfruit
Carica papaya	Papaya
Citrullus vulgaris	Watermelon
Citrus grandis	Pummelo
C. medica	Citron, lemon
C. nobilis	Tangerine
C. reticulata	Mandarin
C. sinensis	Orange
Cydonia oblonga	Quince
Diospyros kaki	Japanese persimmon
Ficus carica	Fig
Fragaria chiloensis	Wild strawberry
F. virginiana	Strawberry
Litchi chinensis	Litchi
Malus asiatica	Chinese apple
M. pumila	Apple
Mangifera indica	Mango
Morus rubra	Red mulberry
Musa cavendishii	Banana
M. paradisiaca	Banana
Passiflora edulis	Passion fruit, purple granadilla
P. ligularis	Passion flower
Persea americana	Avocado pear
Phoenix dactylifera	Date palm
Prunus armeniaca	Apricot
P. avium	Sweet cherry, mazzard
P. cerasifera (divaricata)	Cherry plum
P. cerasus	Cherry
P. domestica	European plum or prune
P. maheleb	Maheleb cherry
P. persica	Peach and nectarine
P. pseudocerasus	Cherry
P. salicinia	Japanese peach
P. serotina	Wild black cherry
Psidium guajava	Guava
Punica granatum	Pomegranate
Pyrus communis	Pear
P. ussuriensis	Chinese pear
Ribes grossularia	Gooseberry
R. nigrum	Black currant
R. rubrum	Red and white currant
Rubus idaeus	European raspberry
R. strigosus	American raspberry
Tamarindus indica	Tamarind

Vaccinium angustifolium	Sugarberry
V. ashei	Rabbiteye blueberry
V. corymbosum	Highbush blueberry
V. myrtillus	Canada blueberry
V. oxycoccos	Cranberry
Viburnum trilobum	American cranberry bush
Vitis labrusca	Fox grape
V. vinifera	Grape

VEGETABLES AND TUBERS

Monocot

Allium ascalonicum	Shallot, green onion
A. cepa	Onion
A. chinense	Onion
A. fistulosum	Welsh onion
A. porrum	Leek
A. sativum	Garlic
A. schoenoprasum	Chives
Asparagus officinalis	Asparagus (var. attilis)
Zea mays	Sweet corn

Dicot

Amoracia lapathifolia	Horseradish
Apium graveolens	Celery
Arabidopsis thaliana	Common wall cress
Beta vulgaris	Sugar, mangold or garden beet
Brassica alboglabra	Chinese kale
B. campestris	Turnip rape
B. carinata	Abyssinian mustard
B. cernua	Karashina
B. chinensis	Chinese mustard or pak-choi
B. hirta	White mustard
B. juncea	Pai, brown mustard, Indian mustard
B. kaber	Charlock
B. napobrassica	Swede or rutabaga
B. napus	Rape, oil rape, kale
B. nigra	Black mustard
B. oleracea	Cole, kale, collards brussels sprouts, cauliflower, cabbage, kohlrabi, broccoli
B. pekinensis	Chinese cabbage or celery cabbage
B. rapa	Turnip
Cajanus cajan (indicus)	Pigeon pea
Canavalia ensiformis	Jack bean
Canna edulis	Edible canna
Capsicum annuum	Common cultivated pepper
C. chinense	Pepper
C. frutescens	Cayenne pepper
C. pendulum	Pepper
C. pubescens	Pepper

Cichorium endivia	Endive
C. intybus	Chicory
Colocasia antiquorum	Taro
Crambe maritima	Sea kale
Cucumis melo	Melon, cantaloupe
C. sativus	Cucumber
Cucurbita ficifolia	Malabar gourd
C. foetidissima	Calabazilla, buffalo gourd
C. maxima	Pumpkin
C. moschata	Winter pumpkin
C. pepo	Summer squash, vegetable marrow
Cynara scolymus	Globe artichoke
Daucus carota	Carrot
Dioscorea alata	Yam
D. batatas	Chinese yam
D. cayennensis	Attoto yam
Eruca sativa Mill.	Rocket salad, rocket or roquette
Ipomea batatas	Sweet potato
Lactuca sativa	Lettuce
Lepidium sativum	Garden cress
Lycopersicon cerasiforme	Cherry tomato
L. esculentum	Tomato
Mahihot utilissima	Manioc, cassava
Nasturtium officinale	Water cress
Pastinaca sativa	Parsnip
Petroselinum crispum (sativum)	Parsley
Physalis peruviana	Ground cherry
Pisum sativum	Pea
Raphanus sativus	Radish
Rheum officinale	Rhubarb
R. rhaponticum	English rhubarb
Scorzonera hispanica	Black salsify
Sechium edule	Chayote
Solanum andigenum	Andean potato
S. melongena	Eggplant
S. muricatum	Pepino
S. phureja	Potato
S. tuberosum	Common potato
Spinacia oleracea	Spinach

NUTS

Dicot

Anacardium occidentale	Cashew
Arachis hypogaea	Peanut
Carya illinoinensis	Pecan
C. ovata	Shagbark hickory
Castanea sativa	Chestnut
Cocos nucifera	Coconut palm
Corylus americana	American hazel, filbert
C. avellana	European hazel, cobnut
Juglans nigra	Black walnut
J. regia	English walnut

J. sinensis	Walnut
Litchi chinensis	Litchi
Macadamia integrifolia	Queensland nut
Pistacia vera	Pistachio nut
Prunus amygdalus	Almond

OIL CROPS (COOKING OR VEGETABLE OILS)

Monocot

Zea mays	Corn

Dicot

Aleurites cordata	Tung, China wood oil
A. moluccana (triloba)	Candlenut
Arachis hypogea	Ground nut, peanut
Brassica campestris	Rapeseed oil, canola oil
B. napus	Rapeseed oil, canola oil
Cannabis sativa	Hempseed oil
Carthamus tinctorius	Safflower oil
Cocos nucifera	Coconut palm
Elaeis guineensis	Oil palm
Glycine gracilus	Manch, soya
G. max	Soybean
G. ussuriensis	Wild soya
Gossypium hirsutum	Cottonseed oil
Helianthus annus	Sunflower
Linum usitatissimum	Flax
Olea europaea	Olive
Papaver somniferum	Poppy seed
Ricinus communis	Castor bean
Sesamum indicum	Sesame

SUGAR CROPS

Monocot

Saccharum officinarum (officinarum x *spontaneum)*	Sugarcane
S. robustum	
S. sinense	Sugarcane
S. spontaneum	Kans grass
Sorghum dochna	Sorgo syrup, sugar sorghum

Dicot

Acer saccharum	Sugar maple
Beta vulgaris	Sugar or mangold beet

FORAGE AND TURF GRASSES

Monocot

Agropyron cristatum	Crested wheatgrass
A. desertorum	Crested wheatgrass
A. elongatum	Tall wheatgrass
A. intermedium	Intermediate wheatgrass
A. smithii	Western wheatgrass
A. spicatum	Blue bunch wheatgrass
A. trachycaulum	Slender wheatgrass
A. trichophorum	Pubescent wheatgrass

Alopecurus pratensis	Meadow foxtail
Andropogon gerardi	Big bluestem
Arrhenatherum elatius	Tall oat grass
Bothriochloa barbinodis	Cane bluestem
B. ischaemum	King ranch bluestem
B. saccharoides	Silver bluestem
Bouteloua curipendula	Side oats grama
B. eriopoda	Black grama
B. gracilis	Blue grama
Bromus erectus	Upright brome
B. inermis	Smooth brome
B. riparius	Meadow brome
Cenchrus ciliaris	Buffel grass
Chloris gayana	Rhodes grass
Cymbopogon nardus	Citronella grass
Cynodon dactylon	Bermuda grass
Dactylis glomerata	Cocksfoot, orchard grass
Dichanthium annulatum	Kleberg bluestem
D. aristatum	Angleton bluestem
D. sericeum	Silky bluestem
Digitaria decumbens	Pangola grass
D. smutsii	
Elymus angustus	Altai wild rye
E. junceus	Russian wild rye
Eragrostis curvula	Weeping love grass
Festuca arundinacea	Tall fescue
F. ovina	Sheeps fescue
F. pratensis	Meadow fescue
F. rubra	Red fescue
Lolium multiflorum	Italian ryegrass
L. perenne	Perennial ryegrass
Panicum maximum	Guinea grass
P. purpurascens	Para grass
P. virgatum	Switchgrass
Paspalum dilatatum	Dallis grass, large water grass
P. notatum	Bahia grass
Pennisetum clandestinum	Kikuyu grass
P. purpureum	Dry napier grass
Phalaris arundinacea	Reed canary grass
Phleum bertolinii	Timothy
P. pratense	Timothy
Poa fendleriana	Mutton grass
P. nemoralis	Wood meadow grass
P. pratensis	Kentucky bluegrass
Setaria sphacelata	Rhodesian timothy
Sorghastrum nutans	Indian grass
Sorghum halepense	Johnson grass
S. sudanense	Sudan grass
Sorghum vulgare	Great millet

FORAGE LEGUMES

Dicot

Coronilla varia	Crown vetch
Crotalaria juncea	Sunn hemp
Lespedeza stipulacea	Korean lespedeza

L. striata	Common lespedeza
L. sericea	
Lotus corniculatus	Birdsfoot trefoil
L. uliginosus	
Lupinus albus	Wolf bean, white lupin
L. angustifolius	Blue lupin
L. luteus	European yellow lupin
L. mutabilis	South American lupin
Medicago arabica	Spotted burr-clover
M. arborea	Tree alfalfa
M. falcata	Yellow lucerne
M. hispida	California burr-clover
M. sativa	Alfalfa
M. tribuloides	Barrel medic
Melilotus albus	White sweet clover
M. officinalis	Yellow sweet clover
Onobrychis viciifolia	Sainfoin
Ornithopus sativus	Serradella
Pueraria thunbergiana	Kudzu vine
Trifolium alexandrinum	Egyptian clover
T. augustifolium	Fineleaf clover
T. diffusum	Rose clover
T. hybridum	Alsike clover
T. incarnatum	Cromson clover
T. ingrescens	Ball clover
T. pratense	Red clover
T. repens	White clover
T. resupinatum	Persian clover
T. subterraneum	Subterranean clover
Trigonella foenum-graecum	Fenugreek
Vicia sativa	Common vetch
V. villosa	Hairy vetch
V. atropurpurea	Purple vetch
V. angustifolia	Narrowleaf vetch
V. dasycarpa	Woolly pod vetch
V. ervilia	Monantha (bitter) vetch
V. pannonica	Hungarian vetch
V. calcarata	Bard vetch

FIBER PLANTS AND WOODS

Monocot

Bambusa vulgaris	Bamboo

Dicot

Agave sisalana	Sisal hemp
Boehmeria nivea	Rhea fiber, ramie
Cannabis indica	Hemp
C. sativa	Hemp
Ceiba pentandra	Silk cotton tree, kapok tree
Corchorus mucronata (striata)	Hemp
Gossypium arboreum	Tree cotton
G. barbadense	Egyptian cotton
G. herbaceum	Cotton

G. hirsutum	Upland cotton
G. nanking	Oriental cotton
Linum angustifolium	Wild flax
L. usitatissimum	Flax
Musa textiles	Manila hemp, abaca

DRUG CROPS

Dicot

Angelica archangelica	Angelica
Chrysanthemum cinerariifolium	Palm pyrethrum
Camellia sinensis	Chinese tea
C. coccineum	Pyrethrum
Coffea arabica	Coffee
C. canephora	Quillow coffee
Cola acuminata	Kola nut
Nicotiana rustica	Tobacco
N. tabacum	Tobacco
Papaver dubium	Poppy
P. somniferum	Opium poppy
Theobroma cacao	Cocoa

SPICES AND FLAVORINGS

Monocot

Vanilla fragrans	Vanilla

Dicot

Artemisa dracunculus	Tarragon
Cinnamomum zeylanicum	Cinnamon tree
Hibiscus esculentus	Okra
Salvia officinalis	Sage
Thymus vulgaris	Thyme
Pimpinella anisum	Anise
Mentha arvensis	Menthol
M. piperita	Peppermint
M. viridis	Spearmint
Coriandrum sativum	Coriander

Table B

Representative Transgenic Plants, Proteinase Inhibitors and Target Pests

Plant	Proteinase Inhibitor	Representative Target Pest
Maize (monocot)	cystatin	*Coleoptera:* *Diabrotica* (corn root worms) *Melanotus, Agriotes, Limonius, Dalopius, Eleodes* (wireworms) *Chaetocnema* (flea beetles) *Macrodactylus* (chafers) *Sphenophorus* (stem weevils) *Sitophilus* (grain weevils) *Oulema* (cereal leaf beetle) *Rhyzopertha, Prostephanus* (grain borers)
	soybean Kunitz trypsin inhibitor, α_1-anti-trypsin, eglin C (Arg 45)	*Lepidoptera:* *Heliothis* (corn earworm) *Ostrinia, Diatraea, Elasmopalpus, Papaipema* (corn borers) *Agrotis, Loxagrotis, Euxoa, Peridroma saucia, Chorizagrotis* (cutworms) *Spodoptera, Pseudaletia* (armyworms) *Chilo, Busseola, Sesamia, Eldana* (stem borers) *Sitotroga cerealella* (grain moth) *Plodia interpunctella* (Indian meal moth) *Coleoptera:* *Phyllophaga, Cyclocephala* (white grubs)

	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above and ***Thysanoptera:*** *Frankliniella, Anaphothrips, Hercothrips* (thrips) ***Homoptera:*** *Dalbulus* (corn stunt leafhoppers) *Cicadulina* (maize streak virus leafhopper) *Rhopalosiphum, Melanaphis* (corn leaf aphid) *Anuraphis* (corn root aphid) *Prosapia* (froghoppers) ***Diptera:*** *Delia platura* (seed corn maggot) *Euxesta* (maize whorl maggot) ***Orthoptera:*** *Melanoplus, Schistocerca, Sphenarium* (grasshoppers) *Aneolamia* (froghoppers) ***Isoptera:*** *Microtermes, Macrotermes, Allodontermes, Odontotermes* (termites) ***Heteroptera:*** *Nezara, Acrosternum, Euschistus* (stinkbugs) *Blissus* (cinchbug) ***Acarina:*** *Tetranychus, Paratetranychus, Oligonychus* (spider mites)
Turf grass (monocot)	cystatin	***Coleoptera:*** *Popillia* (Japanese beetle)
	soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C (Arg 45)	***Coleoptera:*** *Phyllophaga, Cyclocephala* (white grubs) ***Lepidoptera:*** *Crambus* (webworms) *Agrotis, Peridroma saucia, Chorizagrotis* (cutworms)
	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above

Rice (monocot)	cystatin	***Coleoptera:*** *Sitophilus, Lissorhoptrus* (weevils) *Rhyzopertha* (grain borer)
	soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C (Arg 45)	***Lepidoptera:*** *Chilo, Maliarpha, Scirpophaga, Duataea, Elasmopalpus, Sesamia, Rupela* (stemborers) *Mythimna* (armyworm) *Sitroga* (grain moth)
	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above and ***Homoptera:*** *Nilaparvata, Sogatella, Laodelphax* (plant hoppers) *Sogatodes, Nephotettix, Recilan, Cofana, Empoasca* (leafhoppers) ***Diptera:*** *Diopsis* (stalk-eyed fly) *Atherigona, Hydrellia* (maggots) *Orseolia* (gall midge) *Chironomus* (bloodworm) ***Thysanoptera:*** *Stenothrips* (thrips)
Sorghum (monocot)	cystatin	***Coleoptera:*** *Diabrotica* (cucumber beetle) *Melanotus, Agriotes, Dalopius, Eleodes* (wireworms)
	soybean Kunitz trypsin inhibitor, α_1-anti-trypsin, eglin C (Arg 45)	***Lepidoptera:*** *Chilo* (stem borers) *Sesamia, Diatraea, Busseola* (borers) *Spodoptera* (armyworms) *Agrotis, Peridroma saucia, Chorizagrotis* (cutworms) *Heliothis* (corn earworm) *Nola* (webworm)

	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above and ***Homoptera:*** *Poophilus* (spittlebug) *Schizaphis, Rhopalosiphum, Melanaphis, Sipha* (aphids) ***Heteroptera:*** *Blissus* (cinchbug) ***Acarina:*** *Oligonychus* (spider mites) ***Diptera:*** *Atherigona* (shoot fly) *Contarinia* (sorghum midge)
Cotton (dicot)	cystatin	***Coleoptera:*** *Anthonomus* (bollweevil)
	soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C (Arg 45)	***Lepidoptera:*** *Heliothis, Pectinophora* (bollworm) *Spodoptera* (armyworms) *Acontia* (cotton leafworm) *Trichoplusia* (cabbage looper)
	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above and ***Acarina:*** *Oligonychus* (mites) ***Heteroptera:*** *Euschistus* (stinkbug)
Soybean (dicot)	cystatin	***Coleoptera:*** *Zabrotes* (Mexican bean beetle)
	soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C (Arg 45)	***Lepidoptera*** *Anticarsia* (velvet bean caterpillar) *Pseudoplusia* (soybean looper) *Heliothis* (corn earworm) *Spodoptera* (armyworms)

	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above
Tomato (dicot)	cystatin	***Coleoptera:*** *Leptinotarsa* (potato beetle)
	α_1-antitrypsin, soybean Kunitz trypsin inhibitor, eglin C (Arg 45)	***Lepidoptera:*** *Manduca* (Tomato hornworm) *Heliothis* (fruitworm)
	leupeptin, antipain	***Coleoptera*** and ***Lepidoptera*** as above and ***Homoptera:*** *Paratrioza* (psyllid) *Empoasca, Ophiola, Scleroracus, Macrosteles, Circulifer, Ceratagallia, Agallia* (leafhoppers) *Myzus* (aphids)
Potato (dicot)	cystatin	***Coleoptera:*** *Leptinotarsa, Lema* (potato beetle)
	leupeptin, antipain	***Coleoptera*** as above and ***Homoptera:*** *Paratrioza* (psyllid) *Empoasca, Ophiola, Scleroracus, Macrosteles, Circulifer, Ceratagallia, Agallia* (leafhoppers) *Myzus, Macrosiphum, Aphis* (aphids)
Tobacco (dicot)	leupeptin, antipain	***Lepidoptera:*** *Manduca* (Tobacco hornworm) *Heliothis* (Tobacco budworm) ***Homoptera:*** *Ceratagallia, Agallia* (leafhoppers) *Aphis, Myzus* (aphids)

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The claims defining the invention are as follows:

1. A method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein:

(a) said inhibitor is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases with the proviso that the serine proteinase inhibitor is not a member of the Bowman-Birk inhibitor family;

(b) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and

(c) said plant is: (i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or (ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

2. A method of claim 1 wherein the inhibitor originates from, or has substantial sequence homology to a proteinase inhibitor originating from an animal, a bacterium or a fungus or from a plant of a species different from that of the target plant.

3. A method of claim 1 wherein the pest is an insect, an acarid, a fungus or a bacterium.

4. A method of claim 1 wherein the monocot is selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Sugar Crops and Forage and Turf Grasses.

5. A method of claim 4, wherein the monocot is selected from the group of genera consisting of *Avena* (oats), *Hordeum* (barleys), *Oryza* (rice), *Sorghum* (sorghum), *Triticum* (wheats), *Dactylis* (cocksfoot, orchard grass), and *Saccharum* (sugar cane), and *Zea mays* (maize).

6. A method of claim 4 wherein the monocot is selected from the group consisting of *Dactylis* (cocksfoot, orchard grass) and *Zea mays* (maize).

7. A method of claim 1 wherein the dicot is selected from the group of plant types consisting of Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes and Fiber Plants and Woods.

8. A method of claim 7 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales) and *Gossypium* (cottons).

9. A method of claim 7 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes) and *Gossypium* (cottons).

10. A method of claim 1 wherein the proteinase inhibitor is an inhibitor of a serine proteinase.

11. A method of claim 10 wherein the inhibitor is an inhibitor of at least one serine proteinase selected from the group consisting of thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, $C\bar{1}$-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent protease, thermitase, mast cell proteinase I and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio* α-proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, or of a proteinase which has substantial structural or functional similarity to any of these.

12. A method of claim 10 wherein the inhibitor is an inhibitor of trypsin or chymotrypsin, or of a proteinase which has substantial structural or functional similarity to either of these.

13. A method of claim 10 wherein the inhibitor is a member of the soybean Kunitz inhibitor family, the bovine pancreatic trypsin (Kunitz) inhibitor family, the Kazal trypsin inhibitor family, the *Streptomyces* subtilisin inhibitor family, the potato inhibitor I family, the potato inhibitor II family, the α_1-proteinase inhibitor family, the hirudin family, the bdellin family, the eglin family, the inter-α_1 trypsin inhibitor family, the serpin super-family, the $\overline{C1}$-inhibitor family, the *Ascaris* inhibitor family, the leupeptins, the antipains, elastinal and chymostatin, or an inhibitor which has substantial structural or functional similarity to any of these.

14. A method of claim 12 wherein the inhibitor is an inhibitor of chymostrypsin or of a proteinase which has substantial structural or functional similarity to chymotrypsin.

15. A method of claim 14 wherein the inhibitor is a potato I, a potato II, tomato I or tomato II inhibitor.

16. A method of claim 10 wherein the inhibitor is selected from the group consisting of soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C and eglin C mutants.

17. A method of claim 1 wherein the inhibitor is an inhibitor of a thiolproteinase.

18. A method of claim 17 wherein the inhibitor is an inhibitor of papain, bromelain, ficin, calpain, cathepsin B, cathepsin C, cathepsin L, cathepsin H, cathepsin S, chymopapain, clostripain, asclepain, prolyl endopeptidase, pyroglutamyl peptidase, dipeptidyl proteinase I, yeast proteinase B, *Streptococcus* proteinase, *Staphylococcus* thiol proteinase or actinidin, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

19. A method of claim 17 wherein the inhibitor is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64 or a derivative thereof.

20. A method of claim 19 wherein the inhibitor is antipain or leupeptin.

21. A method of claim 19 wherein the inhibitor is selected from the group consisting of egg white cystatin, human cystatin A, human cystatin B, human cystatin C, human cystatin S, rat cystatin α, rat cystatin β and kininogen, L-kininogen and H-kininogen.

22. A method of claim 21 wherein the inhibitor is egg white cystatin.

23. A method of claim 1 wherein the inhibitor is an inhibitor of a metalloproteinase.

24. A method of claim 23 wherein the inhibitor is an inhibitor of carboxypeptidase A, carboxypeptidase B, aminopeptidase, collagenase, calcium-dependent neutral proteinase, thermolysin, angiotensin-converting enzyme, renal dipeptidase, enkephalinase, gelatinase or keratinase, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

25. A method of claim 23 wherein the inhibitor is, or has substantial structural or functional similarity to, a potato carboxypeptidase inhibitor, mammalian collagenase inhibitor, α_2-macroglobulin, tissue bradykinin-potentiating peptide, phosphoramidon, bestatin or amastatin.

26. A method of claim 1 wherein the inhibitor is an inhibitor of an acidic proteinase.

27. A method of claim 26 wherein the inhibitor is an inhibitor of pepsin, rennin, cathepsin D, chymosin, Penicillinopepsin or *Scytalidium* acid protease B, or an inhibitor of a proteinase which have substantial structural or functional similarity to any of these.

28. A method of claim 26 wherein the inhibitor is, or has substantial structural or functional similarity to, pepstatin, *Ascaris* carboxyl proteinase inhibitor, *Bauhinia* pepsin inhibitor, *Scopolia japonica* proteinase inhibitor or potato cathepsin D inhibitor.

29. A method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant, wherein said inhibitor:
(a) is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and
(b) is selected from the group of proteinase inhibitors consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases.

30. A method of claim 29 wherein the inhibitor is selected from the group of non-trypsin proteinase inhibitors consisting of inhibitors of thiol-proteinases, metalloproteinases, acidic proteinases and serine proteinases.

31. A method of claim 29 wherein the plant is a dicot selected from the group of plant types consisting of Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, and Drug Crops.

32. A method of claim 31 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes) *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales), *Gossypium* (cottons) and *Nicotiana* (tobaccos).

33. A method of claim 31 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Gossypium* (cottons), and *Nicotiana* (tobaccos).

34. A method of claim 29 wherein the proteinase inhibitor is a non-trypsin serine proteinase inhibitor.

35. A method of claim 34 wherein the inhibitor is an inhibitor of the serine proteinase chymotrypsin.

36. A method of claim 34 wherein the inhibitor is eglin C or an eglin C mutant.

37. A method of claim 1 which method comprises exposing the pest to a pesticidally effective amount of at least one proteinase inhibitor selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases, in or on the plant, wherein:
(a) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and
(b) said plant is a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods, and Spices and Flavorings.

38. A method of claim 37 wherein said proteinase inhibitor is an inhibitor of a serine proteinase.

39. A method of claim 38 wherein the inhibitor is an inhibitor of trypsin or chymotrypsin, or of a proteinase which has substantial structural or functional similarity to either of these.

40. A method of claim 38 wherein the inhibitor is selected from the group consisting of Soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C and eglin C mutants.

41. A method of claim 37 wherein said proteinase inhibitor is an inhibitor of a thiolproteinase.

42. A method of claim 41 wherein the inhibitor is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64 or a derivative thereof.

43. A method of claim 41 wherein the inhibitor is antipain or leupeptin.

44. A method of claim 41 wherein the inhibitor is egg white cystatin.

45. A method of claim 37 wherein the monocot is maize.

46. A method of claim 37 wherein the pest is a member of the order *Coleoptera* or *Lepidoptera*.

47. A method of claim 46 wherein the pest is selected from the group of genera consisting of *Diabrotica*, *Diatraea*, *Ostrinia* and *Heliothis*.

48. A method of claim 47 wherein the pest is *Diabrotica* or *Ostrinia*.

49. A method of claim 37 wherein the proteinase inhibitor is expressed in the roots, stalks, leaves, seed or pollen of the plant.

50. A method of claim 1 which method comprises exposing the pest to a pesticidally effective amount of at least one proteinase inhibitor selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases, in or on the plant, wherein:
(a) said inhibitor is biologically synthesized in the plant as a result of expression of a foreign gene which encodes the proteinase inhibitor, or as a result of expression of one or more foreign genes encoding one or more precursors of the proteinase inhibitor; and
(b) said plant is a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

51. A method of claim 50 wherein said proteinase inhibitor is an inhibitor of a serine proteinase.

52. A method of claim 51 wherein the inhibitor is an inhibitor of trypsin or chymotrypsin, or of a proteinase which has substantial structural or functional similarity to either of these.

53. A method of claim 51 wherein the inhibitor is selected from the group consisting of Soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C and eglin C mutants.

54. A method of claim 50 wherein said proteinase inhibitor is an inhibitor of a thiolproteinase.

55. A method of claim 54 wherein the inhibitor is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64, or a derivative thereof.

56. A method of claim 54 wherein the inhibitor is antipain or leupeptin.

57. A method of claim 54 wherein the inhibitor is egg white cystatin.

58. A method of claim 50 wherein the dicot is potato, rape, tomato, soybean, pea or cotton.

59. A method of claim 50 wherein the pest is a member of the order *Coleoptera* or *Lepidoptera*.

60. A method of claim 59 wherein the pest is selected from the group of genera consisting of *Diabrotica*, *Diatraea*, *Ostrinia*, *Heliothis*, *Spodoptera* and *Anthonomus*.

61. A method of claim 60 wherein the pest is potato beetle.

62. A method of claim 50 wherein the proteinase inhibitor is expressed in the roots, stalks, leaves, seed or pollen of the plant.

63. A plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:
(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, the proteinase inhibitor being selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and
(b) said plant is:
(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or
(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

64. A plant of claim 63 wherein the monocot is selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Sugar Crops and Forage and Turf Grasses.

65. A plant of claim 64 wherein the monocot is selected from the group of genera consisting of *Avena* (oats), *Hordeum* (barleys), *Oryza* (rice), *Sorghum* (sorghum), *Triticum* (wheats), *Dactylis* (cocksfoot, orchard grass) and *Saccharum* (sugar cane), and *Zea mays* (maize)

66. A plant of claim 64 wherein the monocot is selected from the group consisting of *Zea mays* (maize) and *Dactylis* (cocksfoot, orchard grass).

67. A plant of claim 63 wherein the dicot is selected from the group of plant types consisting of Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes and Fiber Plants and Woods.

68. A plant of claim 67 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales) and *Gossypium* (cottons).

69. A plant of claim 67 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), and *Gossypium* (cottons).

70. A plant of claim 63 wherein the proteinase inhibitor originates from, or has substantial sequence homology to a proteinase inhibitor originating from an animal, a bacterium, a fungus or from a plant of a different species.

71. A plant of claim 63 wherein the proteinase inhibitor is an inhibitor of a serine proteinase.

72. A plant of claim 71 wherein the proteinase inhibitor is an inhibitor of trypsin or chymotrypsin, or an inhibitor of a proteinase which has substantial structural or functional similarity to either of these.

73. A plant of claim 71 wherein the inhibitor is an inhibitor of at least one serine proteinase selected from the group consisting of thrombin, plasmin, elastase, kallikrein, subtilisin, cathepsin G, chymase, acrosin, plasminogen activator, C1-esterase, enterokinase, tryptase, post-proline cleaving enzyme (prolyl endoproteinase), ATP-dependent proteinase, thermitase, mast cell proteinase I and II, *Streptomyces griseus* proteinase A, *Staphylococcus aureus* V8 proteinase, *Tenebrio α* proteinase, urokinase, the blood clotting factors, the complement-activating factors, and the serine carboxypeptidases, or of a proteinase which has substantial structural or functional similarity to any of these.

74. A plant of claim 73 wherein the inhibitor is a member of the Bowman-Birk inhibitor family, the soybean Kunitz inhibitor family, the bovine pancreatic trypsin family, the *Streptomyces* subtilisin inhibitor family, the potato inhibitor I family, the potato inhibitor II family, the

α_1-proteinase inhibitor family, the hirudin family, the bdellin family, the eglin family, the inter-α_1 trypsin inhibitor family, the serpin superfamily, the $C\bar{1}$-inhibitor family, the *Ascaris* inhibitor family, the leupeptins, the antipains, elastinal and chymostatin, or an inhibitor having substantial structural or functional similarity to any of these.

75. A plant of claim 71 wherein the inhibitor is an inhibitor of chymotrypsin or of a proteinase which has substantial structural or functional similarity to chymotrypsin.

76. A plant of claim 75 wherein the inhibitor is a potato I, potato II, tomato I or tomato II inhibitor.

77. A plant of claim 71 wherein the inhibitor is selected from the group consisting of soybean Kunitz trypsin inhibitor, α_1-antitrypsin, eglin C and eglin C mutants.

78. A plant of claim 63 wherein the inhibitor is an inhibitor of a thiolproteinase.

79. A plant of claim 78 wherein the inhibitor is an inhibitor of papain, bromelain, ficin, calpain, cathepsin B, cathepsin C, cathepsin L, cathepsin H, cathepsin S, chymopapain, clostripain, asclepain, prolyl endopeptidase, pyroglutamyl peptidase, dipeptidyl proteinase I, yeast proteinase B, *Streptococcus* proteinase, *Staphylococcus* thiol proteinase or actinidin, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

80. A plant of claim 78 wherein the inhibitor is, or has substantial structural or functional similarity to, a cystatin, calpastatin, bromelain inhibitor, antipain, leupeptin, chymostatin or E64 or a derivative thereof.

81. A plant of claim 80 wherein the inhibitor is antipain or leupeptin.

82. A plant of claim 80 wherein the inhibitor is selected from the group consisting of egg white cystatin, human cystatin A, human cystatin B, human cystatin C, human cystatin S, rat cystatin α, rat cystatin ß and kininogen, L-kininogen and H-kininogen.

83. A plant of claim 80 wherein the inhibitor is egg white cystatin.

84. A plant of claim 63 wherein the inhibitor is an inhibitor of a metalloproteinase.

85. A plant of claim 84 wherein the inhibitor is an inhibitor of carboxypeptidase A, carboxypeptidase B, aminopeptidase, collagenase, calcium-dependent neutral proteinase, thermolysin, angiotensin-converting enzyme, renal dipeptidase, enkephalinase, gelatinase or keratinase or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

86. A plant of claim 84 wherein the inhibitor is, or has substantial structural or functional similarity to, a potato carboxypeptidase inhibitor, mammalian collagenase inhibitor, α_2-macroglobulin, tissue bradykinin-potentiating peptide, phosphoramidon, bestatin or amastatin.

87. A plant of claim 63 wherein the inhibitor is an inhibitor of an acidic proteinase.

88. A plant of claim 87 wherein the inhibitor is an inhibitor of pepsin, rennin, cathepsin D, chymosin, Penicillinopepsin or *Scytalidium* acid protease B, or an inhibitor of a proteinase which has substantial structural or functional similarity to any of these.

89. A plant of claim 87 wherein the inhibitor is, or has substantial structural or functional similarity to, pepstatin, *Ascaris* carboxyl proteinase inhibitor, *Bauhinia* pepsin inhibitor, *Scopolia japonica* proteinase inhibitor or potato cathepsin D inhibitor.

90. A transgenic plant containing a gene capable of expressing a proteinase inhibitor, or a gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:
(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, selected from the group consisting of inhibitors of thiol-proteinases, metalloproteinases, acidic proteinases and non-trypsin serine proteinases; and
(b) said plant is:
(i) a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings; or
(ii) a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, Drug Crops and Spices and Flavorings.

91. A plant of claim 90 wherein said gene or gene group comprises at least one coding section which encodes a non-trypsin proteinase inhibitor, or a precursor of a non-trypsin proteinase inhibitor, selected from the group consisting of inhibitors of thiolproteinases, metalloproteinases, acidic proteinases and serine proteinases.

92. A plant of claim 90 wherein the plant is a dicot selected from the group of plant types consisting of Fruit Crops, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods, and Drug Crops.

93. A plant of claim 92 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Pisum* (peas), *Beta* (beets), *Glycine* (soybean), *Brassica* (rapes and kales), *Gossypium* (cottons), and *Nicotiana* (tobaccos).

94. A plant of claim 92 wherein the dicot is selected from the group of genera consisting of *Lycopersicon* (tomatoes), *Solanum* (potatoes), *Gossypium* (cottons), and *Nicotiana* (tobaccos).

95. A plant of claim 63 containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:
(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and
(b) said plant is a monocot selected from the group of plant types consisting of Cereals, Vegetables and Tubers, Oil Crops, Sugar Crops, Forage and Turf Grasses, Fiber Plants and Woods and Spices and Flavorings.

96. A plant of claim 95 wherein the monocot is maize.

97. A plant of claim 95 wherein the pest is *Diabrotica* or *Ostrinia*.

98. A plant of claim 95 wherein the proteinase inhibitor is expressed in the roots, stalks, leaves, seed or pollen of the plant.

99. A plant of claim 98 wherein the proteinase inhibitor is expressed in the roots of the plant.

100. A plant of claim 63 containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor, wherein:
(a) said gene or gene group comprises at least one coding section which encodes a proteinase inhibitor, or a precursor for a proteinase inhibitor, selected from the group consisting of inhibitors of serine proteinases, thiolproteinases, metalloproteinases and acidic proteinases; and
(b) said plant is a dicot selected from the group of plant types consisting of Cereals, Protein Crops, Fruit Crops, Vegetables and Tubers, Nuts, Oil Crops, Sugar Crops, Forage Legumes, Fiber Plants and Woods and Spices and Flavorings.

101. A plant of claim 100 wherein the dicot is tomato, potato, pea, soybean, rape, or cotton.

102. A plant of claim 100 wherein the pest is potato beetle.

103. A plant of claim 100 wherein the proteinase inhibitor is expressed in the roots, stalks, leaves, seed or pollen of the plant.

104. A DNA sequence coding for chicken egg white cystatin wherein the codons are those preferred in corn.

105. A DNA sequence of claim 104 comprising the DNA sequence of Fig. 9.

106. A vector comprising a DNA sequence coding for a proteinase inhibitor wherein the vector is useful for transformation of plant cells or of *Agrobacterium*.

107. A vector of claim 106 which is a Ti-plasmid derived vector.

108. A vector of claim 106 which is derived from plasmids pCIB10, pCIB710 or pCIB715.

109. A Ti-plasmid-derived vector, substantially as herein described with reference to Example 4.

110. A chimeric gene with CaMV 35S promoter, substantially as herein described with reference to Example 5.

111. A method of controlling a pest which is attacking a target plant, which method comprises exposing the pest to a pesticidally effective amount of a proteinase inhibitor in or on the plant which inhibitor is substantially as herein described with reference to Example 1 or 2.

112. A plant containing a foreign gene capable of expressing a proteinase inhibitor, or a foreign gene or gene group capable of expressing one or more precursors of a proteinase inhibitor which plant is substantially as herein described with reference to any one of Examples 6 to 50.

113. A transgenic plant containing a gene capable of expressing a proteinase inhibitor, or a gene or gene group capable of expressing one or more precursors of a proteinase inhibitor which plant is substantially as herein described with reference to any one of Examples 6 to 50.

DATED this TWENTY FIFTH day of SEPTEMBER 1992

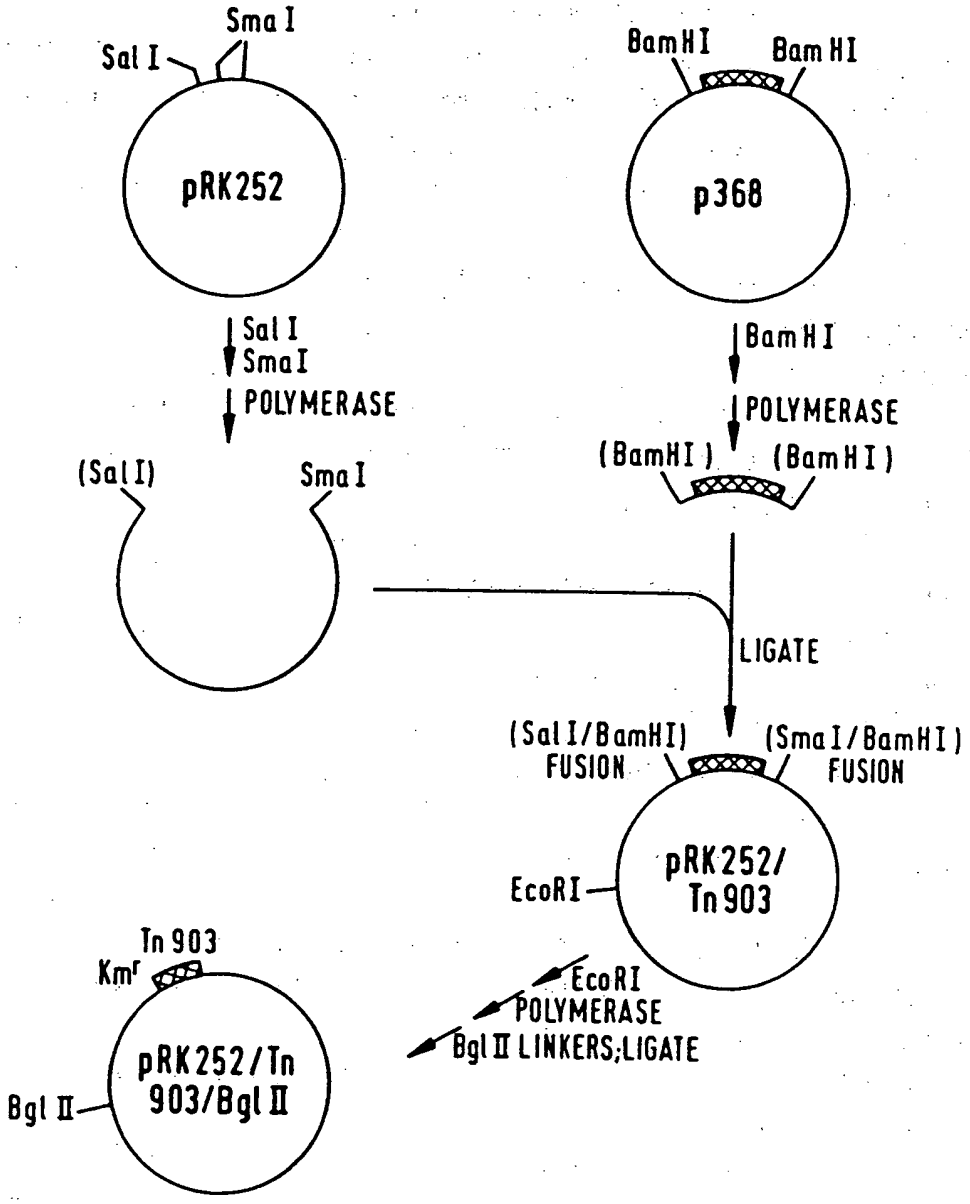
Ciba-Geigy AG

Patent Attorneys for the Applicant

SPRUSON & FERGUSON

amg/1399v

Fig. 1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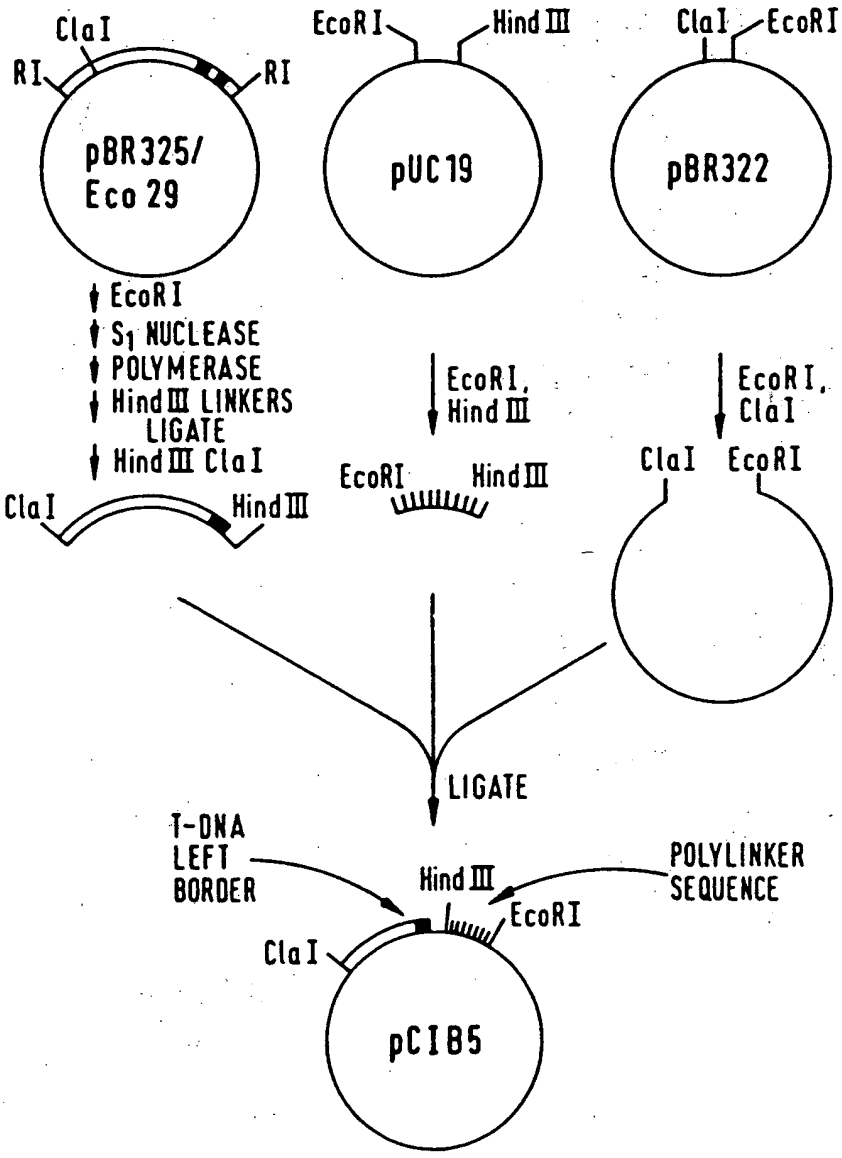
pRK252
BamHI
Bam HI
p368
Sal I
Sma I
POLYMERASE
BamHI
POLYMERASE
(Sal I)
Sma I
(BamHI)
(BamHI)
LIGATE
(Sal I/BamHI)
FUSION
(Sma I/BamHI)
FUSION
pRK252/
Tn 903
EcoRI
EcoRI
POLYMERASE
Bgl II LINKERS;LIGATE
Tn 903
Km^r
pRK252/Tn
903/Bgl II
Bgl II

Fig. 2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RI
RI
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Eco 29
EcoRI
Hind III
pUC 19
ClaI
EcoRI
pBR322
EcoRI
S1 NUCLEASE
POLYMERASE
Hind III LINKERS
LIGATE
Hind III ClaI
ClaI
Hind III
EcoRI,
Hind III
EcoRI
Hind III
EcoRI,
ClaI
ClaI
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T-DNA
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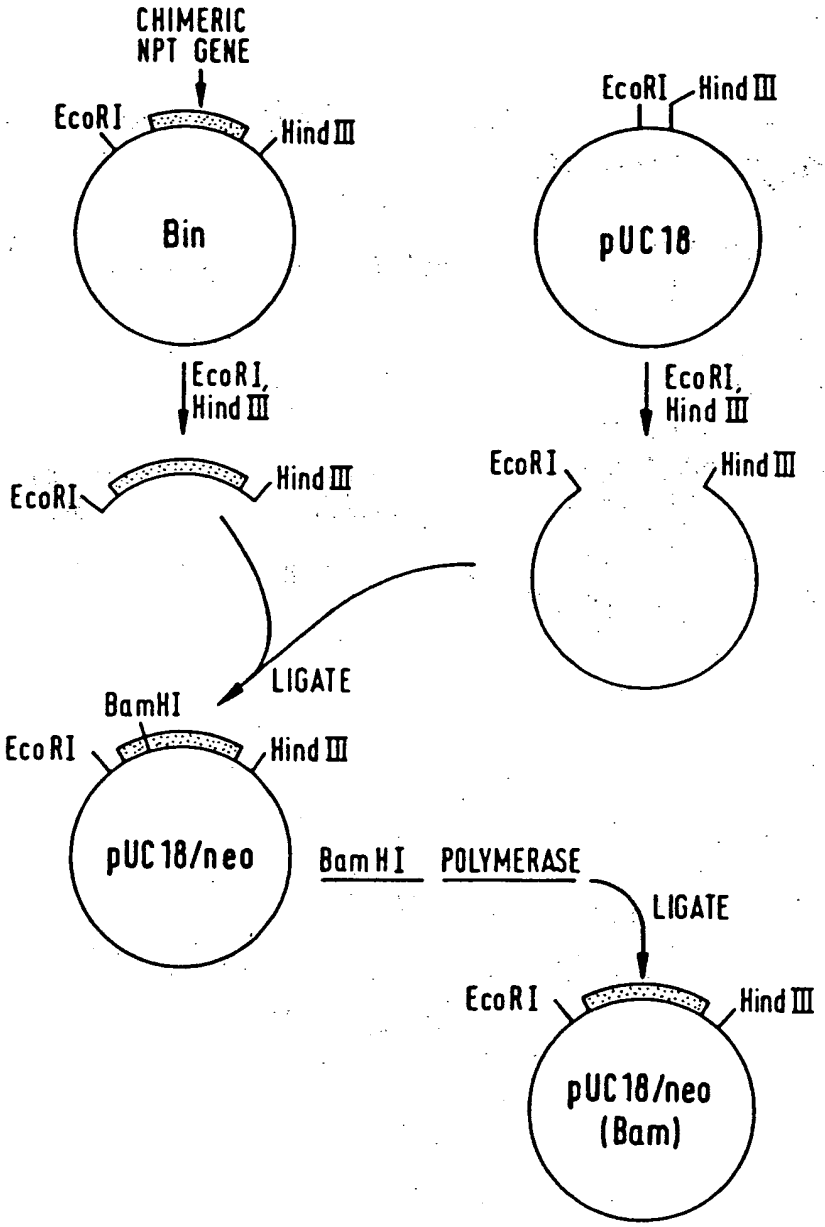
BORDER
Hind III
EcoRI
POLYLINKER
SEQUENCE
ClaI
pCIB5

Fig. 3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Bin
EcoRI
Hind III
pUC 18
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EcoRI,
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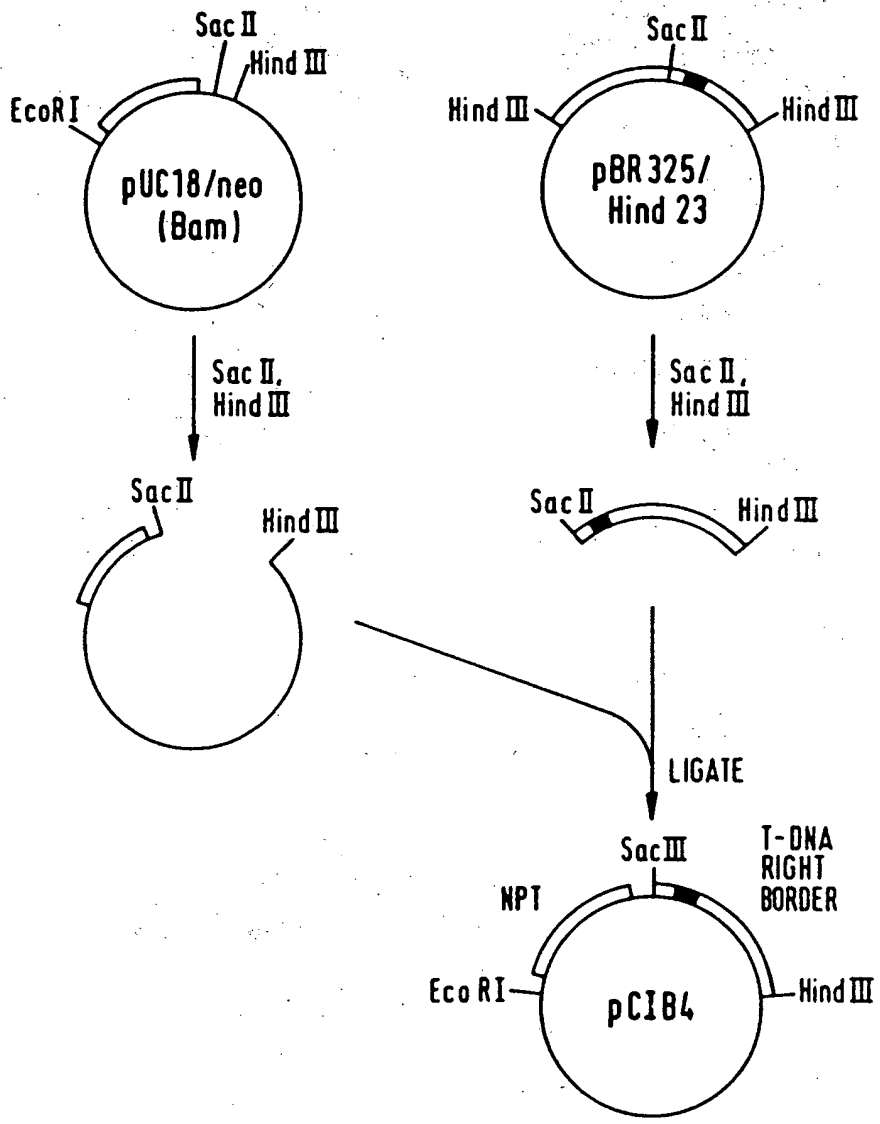
EcoRI
Hind III
EcoRI
Hind III
LIGATE
BamHI
EcoRI
Hind III
pUC 18/neo
Bam HI
POLYMERASE
LIGATE
EcoRI
Hind III
pUC 18/neo
(Bam)

Fig. 4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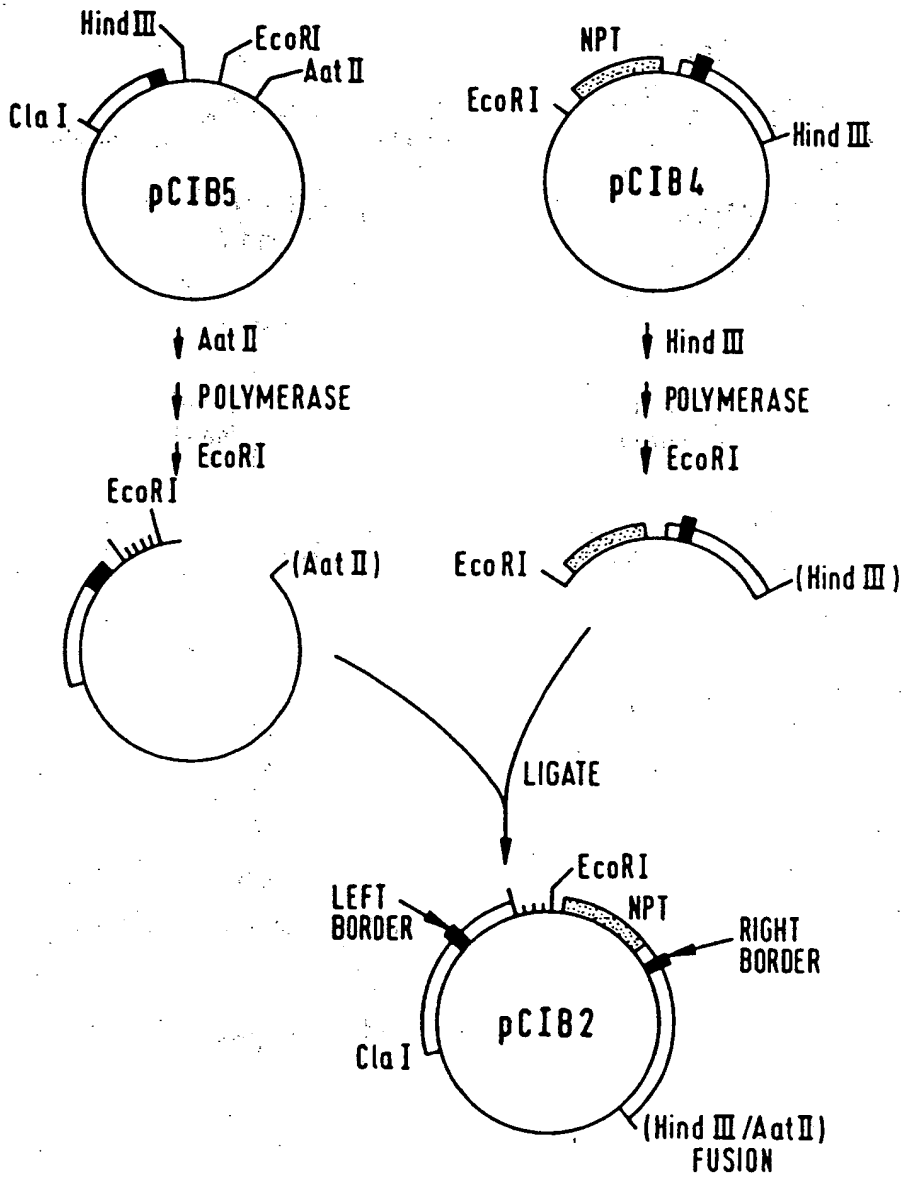
pUC18/neo
(Bam)
Sac II,
Hind III
Sac II
Hind III
Sac II
Hind III
pBR 325/
Hind 23
Sac II,
Hind III
Sac II
Hind III
LIGATE
Sac III
T-DNA
RIGHT
BORDER
NPT
Eco RI
pCIB4
Hind III

Fig. 5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EcoRI
Aat II
Cla I
pCIB5
NPT
EcoRI
Hind III
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Aat II
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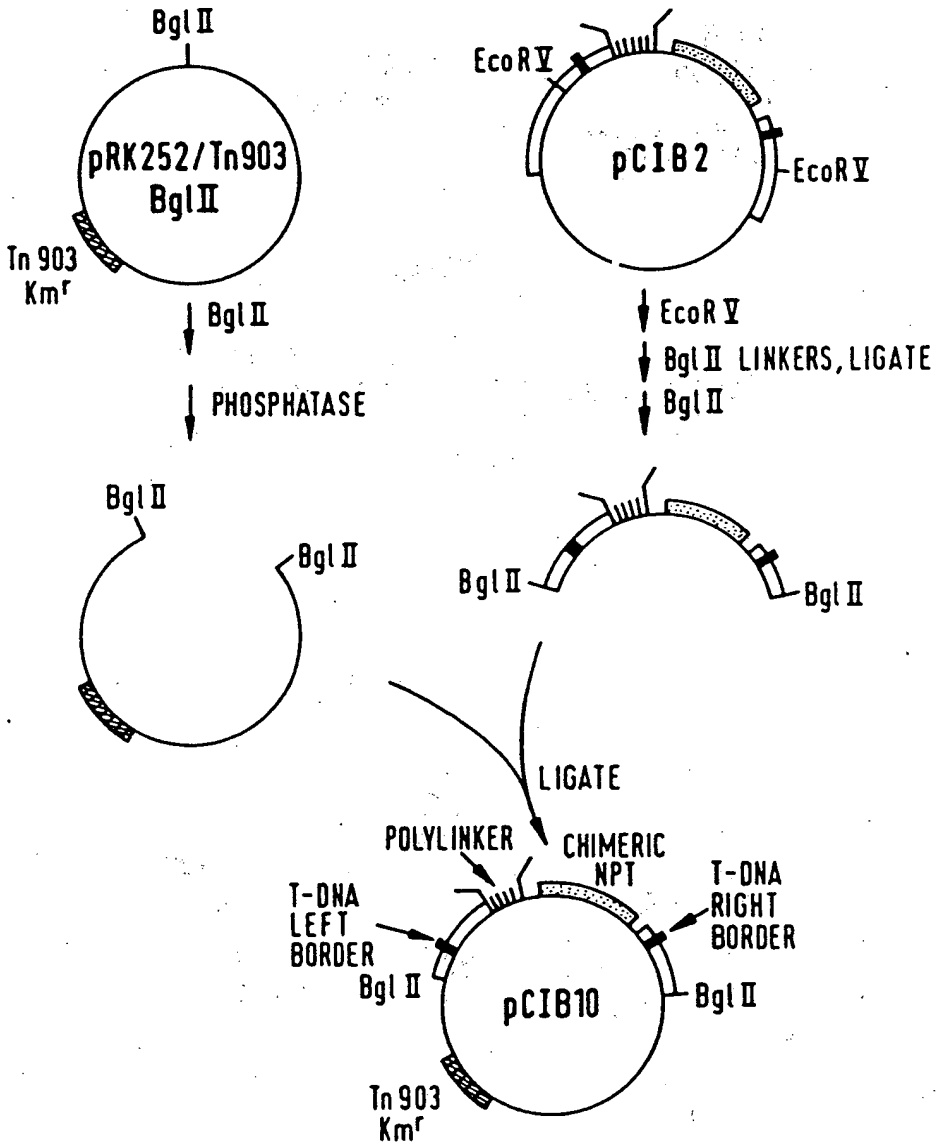
EcoRI
Hind III
POLYMERASE
EcoRI
EcoRI
(Aat II)
EcoRI
(Hind III)
LIGATE
EcoRI
LEFT BORDER
NPT
RIGHT BORDER
pCIB2
Cla I
(Hind III /Aat II) FUSION

Fig. 6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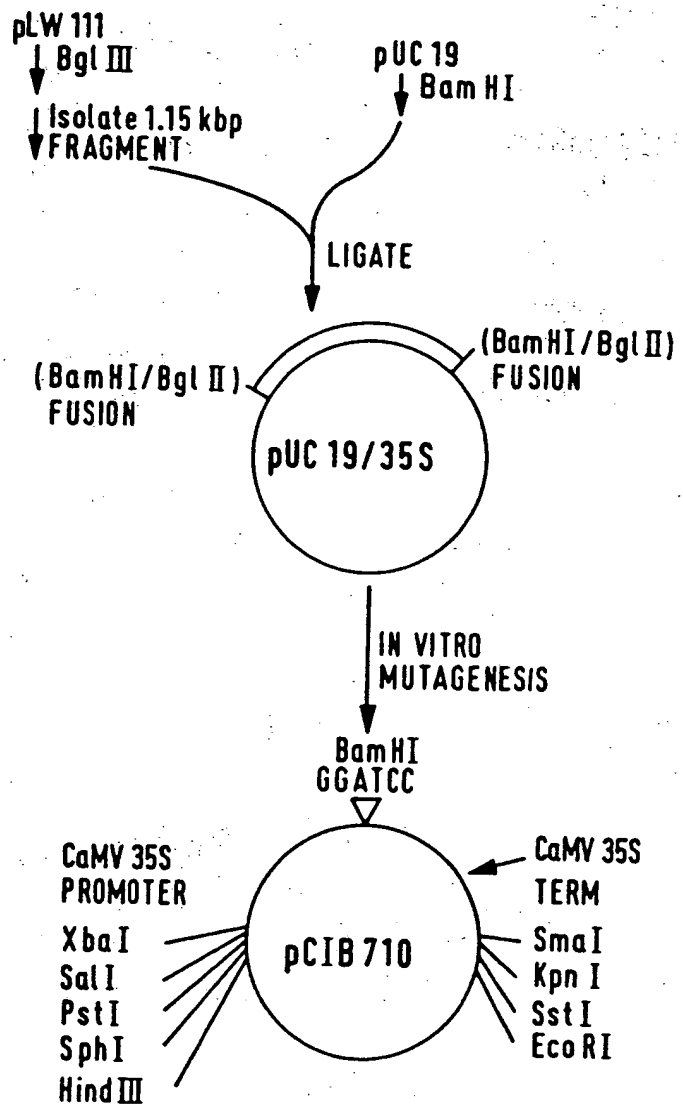
pRK252/Tn903
Bgl II
Tn 903
Km^r
Bgl II
PHOSPHATASE
Bgl II
Bgl II
EcoR V
pCIB2
EcoR V
EcoR V
Bgl II LINKERS, LIGATE
Bgl II
Bgl II
Bgl II
LIGATE
POLYLINKER
CHIMERIC
NPT
T-DNA
LEFT
BORDER
T-DNA
RIGHT
BORDER
Bgl II
pCIB10
Bgl II
Tn 903
Km^r

Fig. 1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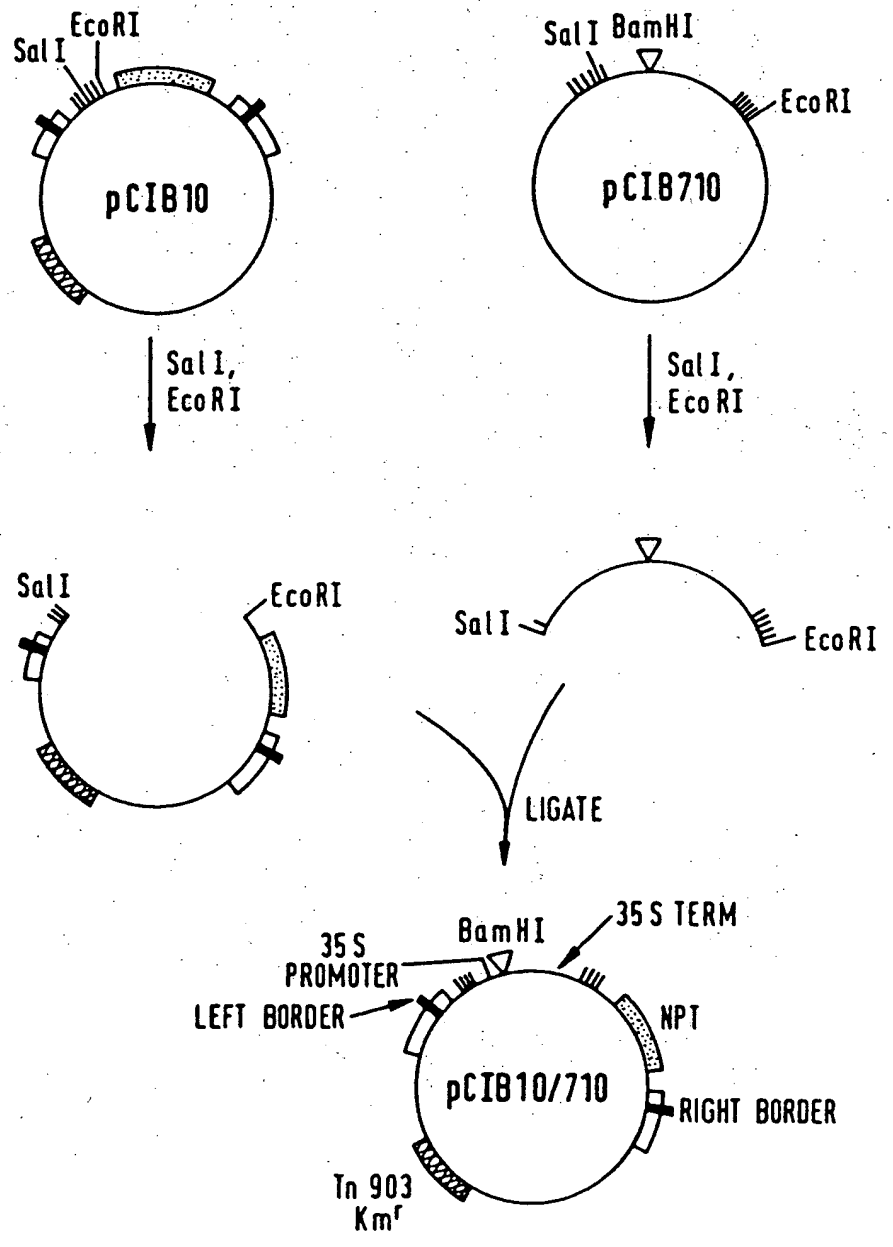
Bgl III
Isolate 1.15 kbp
FRAGMENT
pUC 19
Bam HI
LIGATE
(BamHI/Bgl II)
FUSION
(BamHI/Bgl II)
FUSION
pUC 19/35S
IN VITRO
MUTAGENESIS
BamHI
GGATCC
CaMV 35S
PROMOTER
Xba I
Sal I
Pst I
Sph I
Hind III
pCIB 710
CaMV 35S
TERM
Sma I
Kpn I
Sst I
Eco RI

Fig. 8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EcoRI
pCIB10
SalI, EcoRI
SalI
BamHI
EcoRI
pCIB710
SalI, EcoRI
SalI
EcoRI
SalI
EcoRI
LIGATE
35S TERM
BamHI
35S PROMOTER
LEFT BORDER
NPT
pCIB10/710
RIGHT BORDER
Tn 903 Km^r

Fig. 9

CYSTATIN SEQUENCES

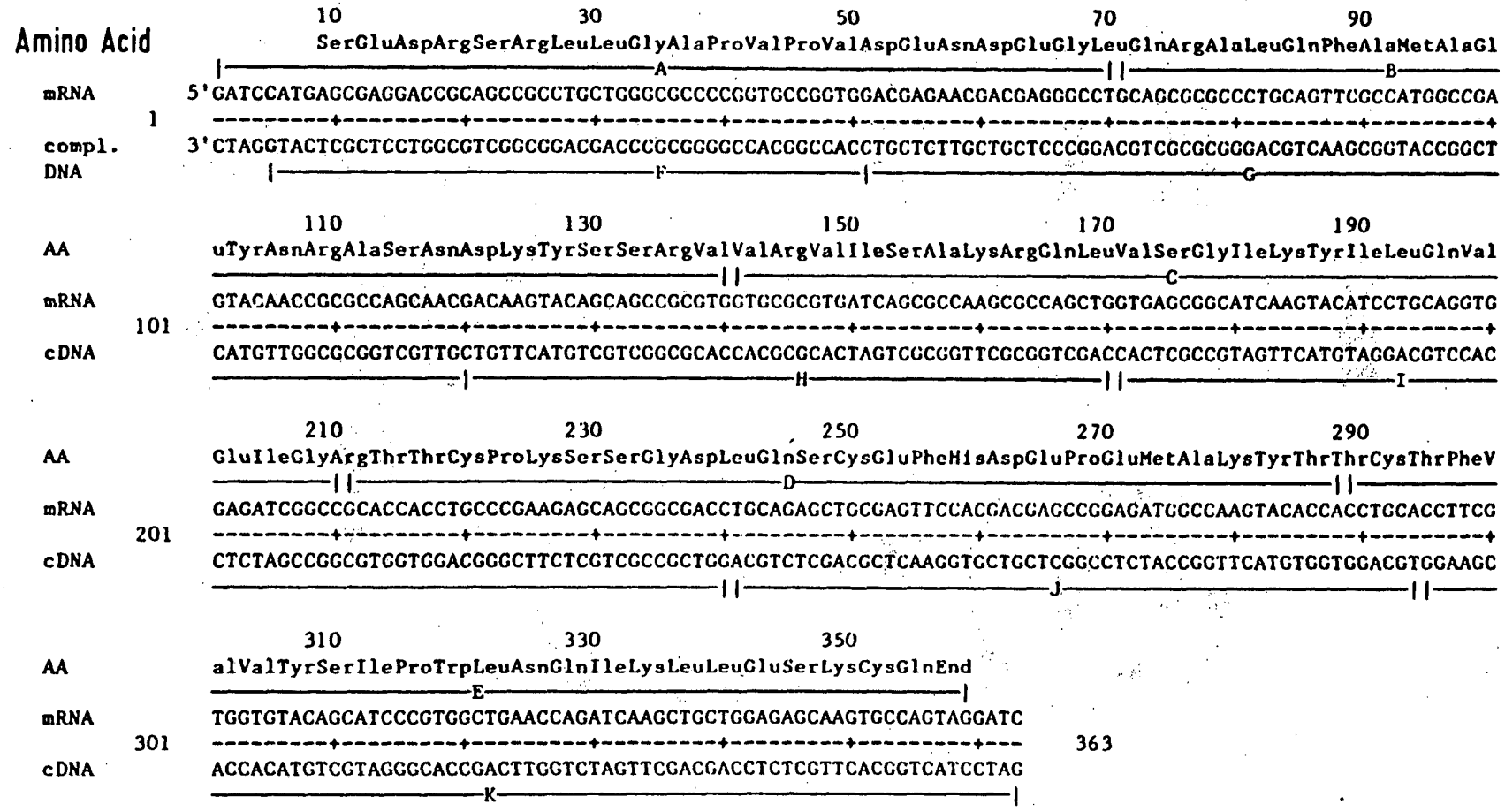

Fig. 10

CYSTATIN GENE FRAGMENTS FOR SYNTHESIS

mRNA strand

A	1- 70	GATCCATGAG CGAGGACCGC AGCCGCCTGC TGGGCGCCCC GGTGCCGGTG GACGAGAACG ACGAGGGCCT
B	71-140	GCAGCGCGCC CTGCAGTTCG CCATGGCCGA GTACAACCGC GCCAGCAACG ACAAGTACAG CAGCCGCGTG
C	141-210	GTGCGCGTGA TCAGCGCCAA GCGCCAGCTG GTGAGCGGCA TCAAGTACAT CCTGCAGGTGGAGATCGGCC
D	211-288	GCACCACCTG CCCGAAGAGC AGCGGCGACC TGCAGAGCTG CGAGTTCCAC GACGAGCCGG AGATGGCCAA GTACACCA
E	289-359	CC TGCACCTTCG TGGTGTACAG CATCCCGTGG CTGAACCAGA TCAAGCTGCT GGAGAGCAAG TGCCAGTAG

Complementary DNA strand

F	5- 50	CACCGG CACCGGGGCG CCCAGCAGGC GGCTGCGGTC CTCGCTCATG
G	51-120	CGTTGC TGGCGCGGTT GTACTCGGCC ATGGCGAACT GCAGGGCGCG CTGCAGGCCC TCGTCGTTCT CGTC
H	121-170	CAGCTG GCGCTTGGCG CTGATCACGC GCACCACGCG GCTGCTGTAC TTGT
I	171-240	GGTCGC CGCTGCTCTT CGGGCAGGTG GTGCGGCCGA TCTCCACCTG CAGGATGTAC TTGATGCCGC TCAC
J	241-294	TGCAGGTGGT GTACTTGGCC ATCTCCGGCT CGTCGTGGAA CTCGCAGCTC TGCA
K	295-362	GATCCTACT GGCACTTGCT CTCCAGCAGC TTGATCTGGT TCAGCCACGG GATGCTGTAC ACCACGAAGG

Fig. 11: SYNTHETIC FRAGMENTS FOR CONSTRUCTING SOYBEAN KUNITZ TYPSIN INHIBITOR GENE

mRNA strand

5M GA TCCATGAAGA GCACCATCTT CTTTGCTCTC TTTCTCTTTT GTGCCTTC

3M GCGTTT GGTGGTGTCT AAGAACAAAC CGTTAGTGGT TCAGTTTCAA AAACTTGATA

AGGAGTCACT CTAG

cDNA strand

3C GATC CTAGAGTGAC TCCTTATCAA GTTTTTGAAA CTGAACCACT AACGGTTTCT

TCTTAGACAC CACCAA

5C GTGAAG GCACAAAAGA GAAAGAGAGC AAAGAAGATG GTGCTCTTCA TGGATC